Clinical relevance of low free protein S levels

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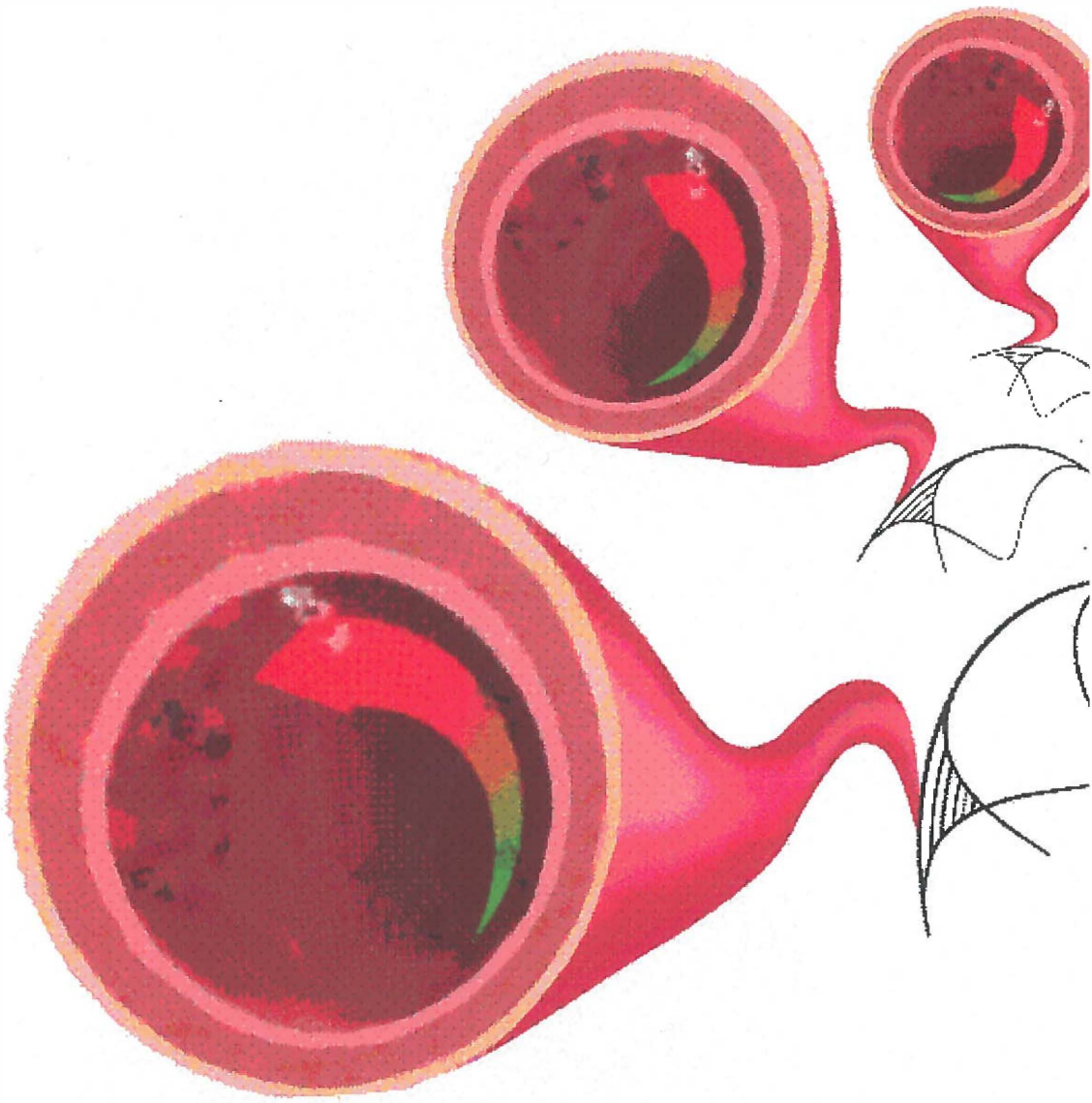
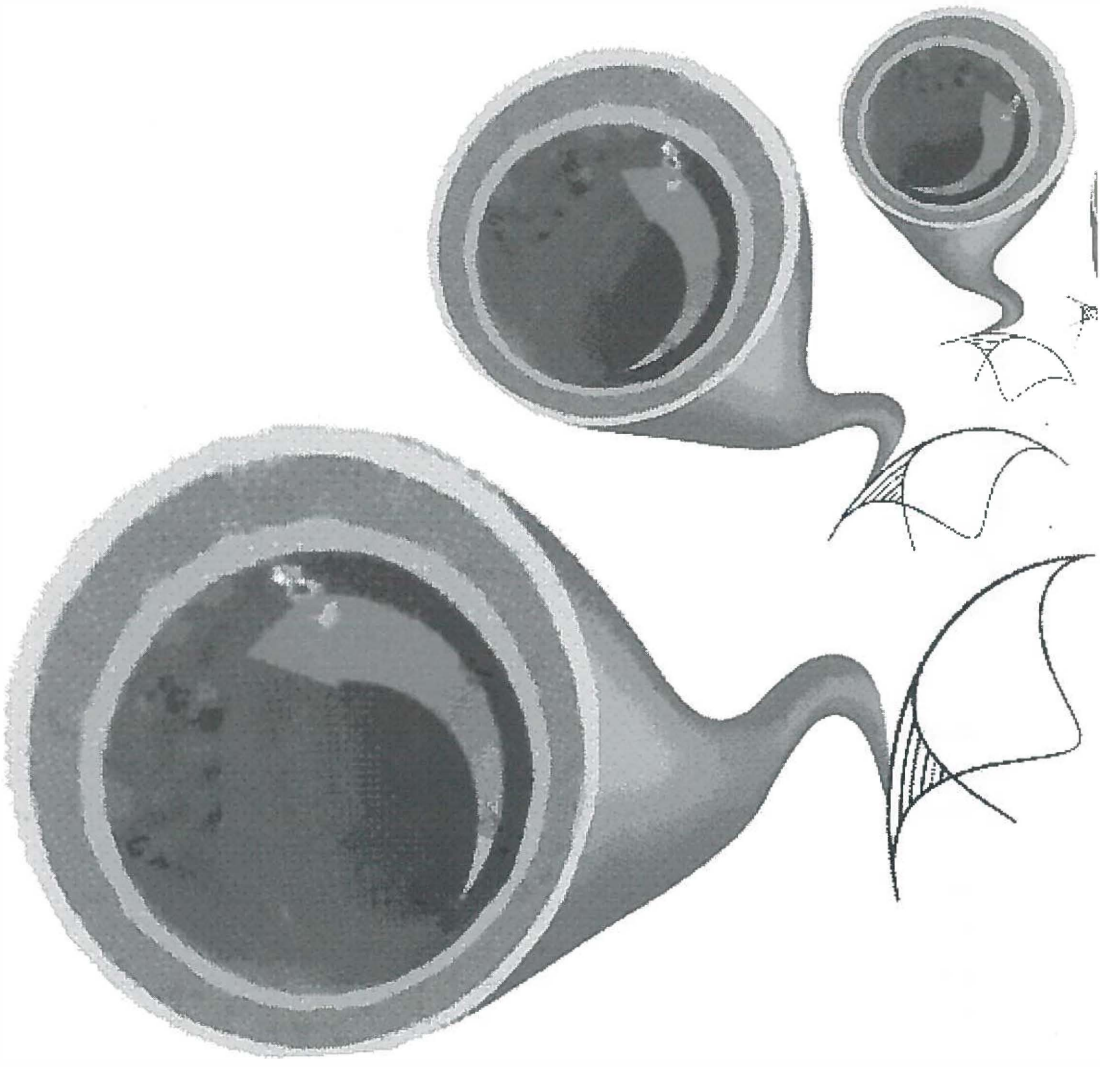


René Mulder

Protein S is a vitamin K-dependent glycoprotein that functions as a cofactor for both activated protein C and Tissue Factor Pathway Inhibitor (TFPI) in the down regulation of coagulation. Hereditary protein S deficiency is a relatively rare coagulation disorder with an estimated prevalence of 0.03 to 0.13% in the general population. Whereas hereditary protein S deficiency predisposes to deep vein thrombosis in the legs or pulmonary embolism, conflicting data have been reported on the risk of thrombosis associated low free protein S levels. Protein S levels are influenced by age, gender, and several acquired conditions. Moreover, protein S assays suffering from low specificity due to interference with many factors, including elevated factor VIII levels and factor V Leiden, causing falsely low protein S levels.

In this context, we performed several studies for risk estimates and accurate detection of protein S levels, in particular free protein S levels.

CLINICAL RELEVANCE OF LOW FREE PROTEIN S LEVELS



René Mulder

Stellingen behorend bij het proefschrift

Clinical relevance of low free protein S levels

1. Free protein S levels below the 5th percentile can identify subjects who are at risk of first venous thrombosis and its recurrence. (*Dit proefschrift*)

2. Free protein S levels below the 5th percentile can identify subjects who are at risk of venous thrombosis in the acute inflammatory setting. (*Dit proefschrift*)

3. In subjects with intermediate low free protein S levels the risk level of venous thrombosis might be attributed by another factor like TFPI. (*Dit proefschrift*)

4. Routinely measuring free protein S levels in case of arterial thrombosis should be discouraged. (*Dit proefschrift*)

5. The use of low cut-off values based on genetically determined protein S deficiency, rather than a reference range based on healthy volunteers can increase the biological performance of protein S assays. (*Dit proefschrift*)

6. Genome-wide association studies are essential for unravelling the genetic architecture of complex traits like low free protein S levels. (PLoS One. 2011;6:e29168.)

7. Moderate coffee consumption is associated with a reduced risk of venous thrombosis. (*J Thromb Haemost. 2011;9:1334-9.*)

8. Impact factors can be a misleading tool in the evaluation of research work. (BMJ. 1997;314:498-502.)

9. The term 'friend' is hollowed out by social media.

10. Doors don't slam open.

11. Its amazing how a 2-years-old boy can give so much meaning to the word 'Dad', simply by varying the tone.

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Dr. H.M. Knol (Marieke)

Aan Amber, Hugo en Kai

"Enjoy when you can, and endure when you must."

Johann Wolfgang von Goethe (1749 - 1832)

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Chapter 1

General introduction

GENERAL INTRODUCTION

Hemostasis

Hemostasis is the overall term denoting the entire process that results in sealing off a leak in blood vessels and restoring normal vascular structure. The hemostatic system is made up of the vascular system, platelets, the coagulation system, and the fibrinolytic system. Hemostasis is divided into a primary and secondary phase. Almost immediately after vascular damage the vessel contracts to reduce blood loss. Platelets circulating in the blood stream recognize the exposed subendothelial structures and attach, thereby forming a platelet plug that services as a platform for coagulation factors. The platelet plug is an irreversible aggregate of platelets that have undergone several morphological and functional changes. This process is called the primary hemostasis and forms the first phase in sealing of the vessel leak. In the second phase the process of coagulation results in a fibrin mesh strengthening and stabilizing the platelet plug. Finally, the clot is removed by the fibrinolytic system after healing of the vessel wall.

Coagulation

The coagulation cascade is a process in which coagulation factors are sequentially enzymatically activated. The classical concept of coagulation was presented about half a century ago (1, 2). This concept suggested that coagulation is initiated by two different pathways. The intrinsic pathway involved coagulation factors XII, XI, IX, VIII, and the extrinsic pathway involved tissue factor and factor VII. It was thought that both pathways converged on a common pathway to activate factor X, leading to formation of thrombin (factor IIa), which converts soluble fibrinogen to unsoluble fibrin.

New insights into the mechanism of coagulation have proposed a modification of the classical cascade to a more all-encompassing concept and is depicted in Figure 1 (3-6).

Coagulation is initiated by the exposure of tissue factor to plasma. The exposed tissue factor interacts with factor VII and forms the tissue factor-factor VIIa complex. Subsequently small amounts of factor IXa and factor Xa are produced. The generation of factor Xa results in production of thrombin which is further boosted through activation of factors IX and XI and cofactors V and VIII. Ultimately, thrombin converts soluble fibrinogen to unsoluble fibrin. In addition, thrombin together with cofactor thrombomodulin (TM) inhibit fibrinolysis of the clot by activation of thrombin activatable fibrinolysis inhibitor (TAFI).

Natural inhibitors of coagulation

The coagulation system is carefully regulated by natural inhibitors of coagulation, like antithrombin, tissue factor pathway inhibitor (TFPI), protein C and protein S (Figure 1). Deficiency of one of these inhibitors lead to excessive blood clotting (thrombophilia).

Antithrombin (AT) is a serine protease inhibitor that inhibits thrombin (factor IIa), factor VIIa, factor IXa and factor Xa, factor XIa, and factor XIIa (7-12). The activity of antithrombin is dramatically accelerated in the presence of heparin, due to a conformational change exposing the reactive site (13).

TFPI is a protein with a highly negatively charged NH_2-terminal followed by three tandem Kunitz-type serine proteinase inhibitor domains, and a highly positively charged carboxy-terminal region (14). TFPI inhibits factor Xa, and, bound to factor Xa, results in the inhibition of the tissue factor-factor VIIa complex (15).

Protein C circulates as an inactive serine protease. It is activated (APC) on the endothelial cell surface by thrombin complexed with thrombomodulin (16). The endothelial protein C receptor further stimulates this activation (17). APC complexes with cofactor protein S in the proteolytic degradation of activated factors Va and VIIIa (18-21).

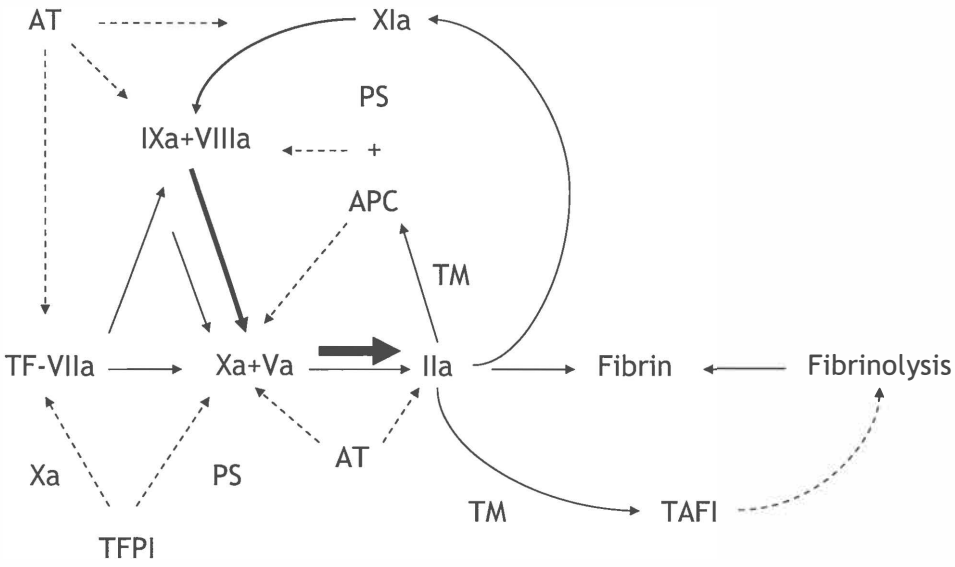

Figure 1. A revised model of the blood coagulation. With permission of the authors this figure was used and modified (6). Abbreviations used: TF-VIIa, tissue factor-factor VIIa complex; TFPI, tissue factor pathway inhibitor; AT, antithrombin; Xa+Va, factor Xa and Va (depicting the prothrombinase complex); IXa+VIIIa, factor IXa and factor VIIIa (depicting the tenase complex); IIa, thrombin; APC, activated protein C; PS, protein S; TM, thrombomodulin; TAFI, thrombin activatable fibrinolysis inhibitor; XIa, factor XIa. Uninterrupted lines indicate activation and interrupted lines indicate inhibition.

Protein S

In 1977, Di Scipio et al. reported a newly discovered vitamin K-dependent human plasma protein called protein S (22). In contrast to most other plasma vitamin K-dependent proteins, protein S is not a serine protease enzyme, but exhibits APC-dependent and APC-independent cofactor activities (18-21, 23).

Protein S is mainly synthesized in the liver, but also in endothelial cells, megakaryocytes, Leydig cells, osteoblasts, and vascular smooth muscle cells (24).

In plasma, protein S is present in two forms (Figure 2). Most protein S is bound to the ß-chain of complement component C4b-binding protein (C4BPß) (25). The remaining 30-40% of protein S is free in plasma and possesses most of the cofactor activity for APC (26). The importance of this cofactor activity is supported by an association between protein S deficiency and venous thrombosis (27-29).

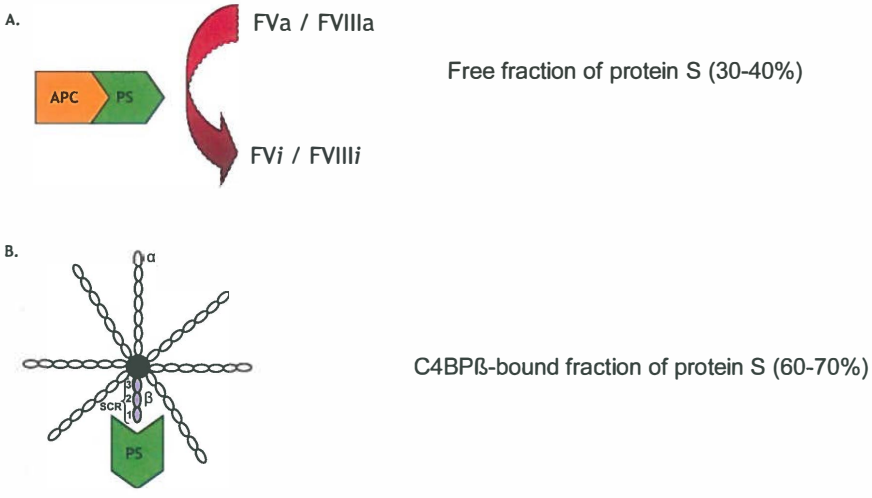

Figure 2. In human plasma, a fraction of protein S (PS) circulates in free form (A), that functions as cofactor for activated protein C (APC) in the inhibition (i) of activated factors (F)Va and VIIIa. The remaining fraction of PS (B) is bound to the first short consensus repeat (SCR) in the ß-chain of complement component C4-binding protein (C4BP).

Structure of protein S

Mature protein S contains 676 amino acids in eight domains (Figure 3). Vitamin K-dependent proteins, like protein S are characterized by post-translation modification of glutamic acid residues to γ-carboxyglutamic acid (Gla) residues (30). The Gla-domain is necessary for binding to negatively charged phospholipid membranes in a Ca^{2+}-dependent manner (31). The Gla-domain is followed by a thumb loop that interacts with APC (32). Protein S further contains 4 epidermal growth factor-like (EGF) domains. In particular EGF domains 2 to 4 are important for protein S cofactor activity

(33). Moreover, it has recently been suggested that residues within the Gla and EGF1 domains of protein S act cooperatively for its APC cofactor function (34). At the carboxyl-terminal end there are 2 identical laminin domains forming the socalled sex hormone-binding globulin (SHBG) domain (35). This domain contains a high affinity binding site for the C4BPß (36-38). C4BPß contains three short consensus repeats (SCR) and a carboxyl-terminal region with two cysteine residues that probably form interchain disulfide bridges (39). Fernandez et al. demonstrated that the protein S binding site on C4BPß involves the first SCR (40). The second SCR was found to contribute to the interaction of C4BPß with protein S (41).

For the molecular background of protein S deficiency, it is necessary to know the location and particular effects of mutations occurring in the protein S locus. The protein S locus is situated near the centromer of chromosome 3 of the human genome, and contains two highly homologous genes; the active gene, PROS1, and an inactive pseudogene, PROSP (42). The PROS1 gene contains 15 exons and 14 introns spanning more than 80 kDa (43).

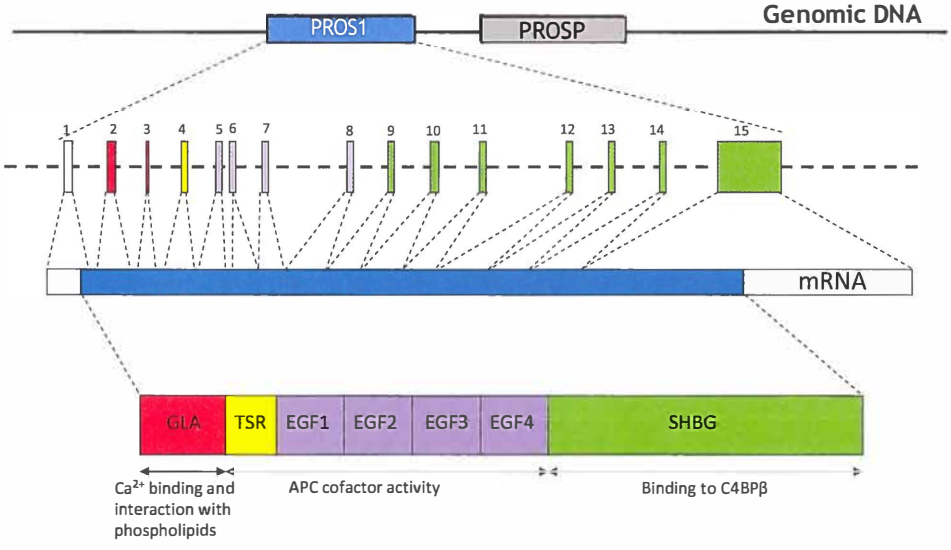

Figure 3. Schematic representation of domain structure of protein S. Abbreviations used for domains: γ-carboxyglutamic acid (GLA), thrombin sensitive region (TSR), epidermal growth factor-like (EGF) and sex hormone-binding globulin (SHBG).

APC-dependent functions of protein S

Protein S exhibits APC cofactor activity in the proteolytic degradation of activated factors Va and VIIIa (18-21). APC-dependent inactivation of factor Va in the presence of protein S results in enhanced cleavage at Arg306 and subsequently at Arg506, leading to a rapid inactivation of factor Va activity (44, 45). The exact mechanism responsible for this inactivation is not completely understood, but may be partially brought about through relocation of the active site of APC, resulting in an optimal exposure to the Arg306 cleavage site of factor Va (46). Although free protein S is considered to possess most of APC cofactor activity, this is also observed for protein S complexed with C4BPß (47).

In the presence of both protein S and factor V, the APC-dependent inactivation of activated factor VIIIa occurs through cleavage at Arg336 and Arg562 (48, 49).

APC-independent functions of protein S

More recently, protein S has also been identified as cofactor for TFPI in the inhibition of factor Xa (23). The exact mechanism by which protein S exhibits cofactor activity for TFPI is not yet unravelled. Ndonwi et al. demonstrated that the Kunitz-3 domain of TFPI is required for complexation with protein S (50).

Other functions of protein S

In addition to cofactor activities for the coagulation inhibitors APC and TFPI, protein S also enhances fibrinolysis by inactivating plasminogen activator inhibitor (51) and decreasing thrombin-activatable fibrinolytic inhibitor (52).

Protein S deficiency

Based on plasma levels of protein S, protein S deficiency can be classified into three categories (Table 1) (53). In type I protein S deficiency both total and free protein S antigen levels are reduced. Type III protein S

deficiency is characterized by only decreased free protein S antigen levels. These two phenotypes are considered to be quantitative forms of protein S deficiency and the most abundantly occuring forms (95%). Type II protein S deficiency, also known as a functional defect or qualitative form of protein S deficiency, is characterized by impaired function of protein S with normal antigen levels.

Unfortunately, accurately measuring protein S levels is notoriously difficult due to variations in protein S levels related to age, gender, and acquired conditions (54-60). Moreover, protein S levels can be quantified using various immunological and activity protein S assays, each with there own sensitivity and specificity profile. For instance, protein S activity assays suffer from low specificity due to interference with elevated factor VIII levels and factor V Leiden, causing falsely low protein S levels (61).

Table 1. Classification of protein S deficiency

Type	TPS	PSact	FPS
I	↓	↓	↓
II	N	↓	N
III	N	↓	↓

According to the International Society on Thrombosis and Haemostasis (ISTH) protein S deficiency is categorized based on plasma levels of total protein S antigen levels (TPS), free protein S antigen levels (FPS) and APC cofactor activity (PSact) (53).

Clinical relevance of protein S deficiency

Protein S deficiency can be hereditary or acquired. Hereditary protein S deficiency is an autosomal dominant disorder with both heterozygous and homozygous genetic expression. Homozygous and compound heterozygous protein S deficiencies have been rarely reported, and are associated with severe purpura fulminans in the neonatal period (62). To date, more than 200 mutations in the PROS1 gene are described (63). Among the PROS1 mutations, the most common mutation is a serine to proline mutation at

codon 460 (S460P) in exon 13, causing the protein S Heerlen variant (64-66). Although this mutation is associated with a type III protein S deficiency (67), it is still under debate whether or not increased risk of venous thrombosis is observed in subjects carrying this mutation (66, 68).

Hereditary protein S deficiency is considered as a risk factor for venous thrombosis (27-29) with a low prevalence of 0.03 to 0.13% in the general population and 1-13 % in patients with venous thrombosis (69, 70). In line with these data, some studies failed to show an increased risk of venous thrombosis in association with low free protein S levels (71-72). In addition, whether low protein S levels can be used to identify subjects who are at risk for arterial thrombosis is uncertain. Most information on this area comes from only small case studies (73-76).

Acquired protein S deficiency has been reported during many conditions, including use of oral anticoagulant therapy, pregnancy, nephrotic syndrome, auto-immune disorders, and infections such as human immunodeficiency virus (54-57). The cause and clinical relevance of reduced protein S levels, in particular low free protein S antigen levels, during these acquired conditions is not yet fully understood.

Aims of the study

In the current thesis we present several studies for risk estimates and accurate detection of protein S levels, in particular free protein S levels.

In **chapter 2** we describe a retrospective family cohort study to define an optimal cut-off value for free protein S to identify relatives at risk of venous thrombosis.

In **chapter 3** we investigated in a case-control study whether decreased free protein S antigen levels are associated with venous thrombosis during the acute setting at the emergency department. Futhermore, we assessed whether the effects of preceding episodes of infectious symptoms, increased C-reactive protein concentrations and increased total C4BP levels attenuated the results.

In **chapter 4** we assessed whether low free protein S levels and high factor VIII levels were associated with arterial thrombosis in a retrospective cohort study from a large series of families with thrombophilic defects.

In **chapter 5** we evaluated the diagnostic performance of nine different protein S assays, using cut-off values based on protein S levels in healthy volunteers and genetically-confirmed heterozygous PROS1 mutation carriers.

In **chapter 6** we investigated a possible association between free TFPI levels and the relatively low risk of venous thrombosis in subjects carrying the protein S Heerlen variant.

REFERENCES

1. Davie EW, Ratnoff OD. Waterfall sequence for intrinsic blood clotting. Science. 1964;145:1310-2.
2. MacFarlane RG. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. Nature. 1964;202:498-9.
3. Naito K, Fujikawa K. Activation of human blood coagulation factor XI independent of factor XII. Factor XI is activated by thrombin and factor XIa in the presence of negatively charged surfaces. J Biol Chem. 1991;266:7353-8.
4. Gailani D, Broze GJ Jr. Factor XI activation in a revised model of blood coagulation. Science. 1991;253:909-12.
5. Von dem Borne PA, Meijers JC, Bouma BN. Feedback activation of factor XI by thrombin in plasma results in additional formation of thrombin that protects fibrin clots from fibrinolysis. Blood. 1995;86:3035-42.
6. Bouma BN, von dem Borne PA, Meijers JC. Factor XI and protection of the fibrin clot against lysis--a role for the intrinsic pathway of coagulation in fibrinolysis. Thromb Haemost. 1998;80:24-7.
7. Abildgaard U. Highly purified antithrombin 3 with heparin cofactor activity prepared by disc electrophoresis. Scand J Clin Lab Invest. 1968;21:89-91.
8. Lawson JH, Butenas S, Ribarik N, Mann KG. Complex-dependent inhibition of factor VIIa by antithrombin III and heparin. J Biol Chem. 1993;268:767-70.
9. Rosenberg JS, McKenna PW, Rosenberg RD. Inhibition of human factor IXa by human antithrombin. J Biol Chem. 1975;250:8883-8.
10. Marciniak E. Factor-Xa inactivation by antithrombin 3. Evidence for biological stabilization of factor Xa by factor V-phospholipid complex. Br J Haematol. 1973;24:391-400.

11. Scott CF, Colman RW. Factors influencing the acceleration of human factor XIa inactivation by antithrombin III. Blood. 1989;73:1873-9.

12. Stead N, Kaplan AP, Rosenberg RD. Inhibition of activated factor XII by antithrombin-heparin cofactor. J Biol Chem. 1976;251:6481-8.

13. Rosenberg RD, Damus PS. The purification and mechanism of action of human antithrombin-heparin cofactor. J Biol Chem. 1973;248:6490-505.

14. Wun TC, Kretzmer KK, Girard TJ, Miletich JP, Broze GJ Jr. Cloning and characterization of a cDNA coding for the lipoprotein-associated coagulation inhibitor shows that it consists of three tandem Kunitz-type inhibitory domains. J Biol Chem. 1988;263:6001-4.

15. Broze GJ Jr, Warren LA, Novotny WF, Higuchi DA, Girard JJ, Miletich JP. The lipoprotein-associated coagulation inhibitor that inhibits the factor VII-tissue factor complex also inhibits factor Xa: insight into its possible mechanism of action. Blood.1988;71:335-43.

16. Esmon CT. The protein C pathway. Chest. 2003;124(3 Suppl):26S-32S.

17. Esmon CT. The endothelial cell protein C receptor. Thromb Haemost. 2000;83:639-43.

18. Walker FJ. Regulation of activated protein C by a new protein. A possible function for bovine protein S. J Biol Chem. 1980;255:5521-4.

19. Walker FJ. Regulation of activated protein C by protein S. The role of phospholipid in factor Va inactivation. J Biol Chem. 1981;256:11128-31.

20. Walker FJ. Protein S and the regulation of activated protein C. Semin Thromb Hemost. 1984;10:131-8.

21. Walker FJ, Chavin SI, Fay PJ. Inactivation of factor VIII by activated protein C and protein S. Arch Biochem Biophys. 1987;252:322-8.

22. Di Scipio RG, Hermodson MA, Yates SG, Davie EW. A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and protein S. Biochemistry. 1977;16:698-706.

23. Hackeng TM, Seré KM, Tans G, Rosing J. Protein S stimulates inhibition of the tissue factor pathway by tissue factor pathway inhibitor. Proc Natl Acad Sci U S A. 2006;103:3106-11.

24. Dahlbäck B. Protein S and C4b-binding protein: components involved in the regulation of the protein C anticoagulant system. Thromb Haemost. 1991;66:49-61.

25. Härdig Y, Rezaie A, Dahlbäck B. High affinity binding of human vitamin K-dependent protein S to a truncated recombinant beta-chain of C4b-binding protein expressed in Escherichia coli. J Biol Chem. 1993;268:3033-6.

26. Dahlbäck B. The tale of protein S and C4b-binding protein, a story of affection. Thromb Haemost. 2007;98:90-6.

27. Comp PC, Nixon RR, Cooper MR, Esmon CT. Familial protein S deficiency is associated with recurrent thrombosis. J Clin Invest. 1984;74:2082-8.

28. Comp PC, Esmon CT. Recurrent venous thromboembolism in patients with a partial deficiency of protein S. N Engl J Med. 1984;311:1525-8.

29. Schwarz HP, Fischer M, Hopmeier P, Batard MA, Griffin JH. Plasma protein S deficiency in familial thrombotic disease. Blood. 1984;64:1297-300.

30. Furie B, Bouchard BA, Furie BC. Vitamin K-dependent biosynthesis of gamma-carboxyglutamic acid. Blood. 1999;93:1798-808.

31. Stenflo J. Contributions of Gla and EGF-like domains to the function of vitamin K-dependent coagulation factors. Crit Rev Eukaryot Gene Expr. 1999;9:59-88.

32. Lu D, Xie RL, Rydzewski A, Long GL. The effect of N-linked glycosylation on molecular weight, thrombin cleavage, and functional activity of human protein S. Thromb Haemost. 1997;77:1156-63.

33. Dahlbäck B, Hildebrand B, Malm J. Characterization of functionally important domains in human vitamin K-dependent protein S using monoclonal antibodies. J Biol Chem. 1990;265:8127-35.

34. Ahnström J, Andersson HM, Canis K, Norstrøm E, Yu Y, Dahlbäck B, Panico M, Morris HR, Crawley JT, Lane DA. Activated protein C cofactor function of protein S: a novel role for a γ-carboxyglutamic acid residue. Blood. 2011;117:6685-93.

35. Giri TK, Villoutreix BO, Wallqvist A, Dahlbäck B, de Frutos PG. Topological studies of the amino terminal modules of vitamin K-dependent protein S using monoclonal antibody epitope mapping and molecular modeling. Thromb Haemost. 1998;80:798-804.

36. Villoutreix BO, Dahlbäck B, Borgel D, Gandrille S, Muller YA. Three-dimensional model of the SHBG-like region of anticoagulant protein S: new structure-function insights. Proteins. 2001;43:203-16.

37. Giri TK, Linse S, Garcia de FP, Yamazaki T, Villoutreix BO, Dahlbäck B. Structural requirements of anticoagulant protein S for its binding to the complement regulator C4b-binding protein. J Biol Chem. 2002;277:15099-106.

38. Evenas P, Garcia de FP, Linse S, Dahlbäck B. Both G-type domains of protein S are required for the high-affinity interaction with C4b-binding protein. Eur J Biochem. 1999;266:935-42.

39. Hillarp A, Dahlbäck B. Cloning of cDNA coding for the beta chain of human complement component C4b-binding protein: sequence homology with the alpha chain. Proc Natl Acad Sci U S A. 1990;87:1183-7.

40. Fernández JA, Griffin JH. A protein S binding site on C4b-binding protein involves beta chain residues 31-45. J Biol Chem. 1994;269:2535-40.

41. Van de Poel RH, Meijers JC, Bouma BN. Interaction between protein S and complement C4b-binding protein (C4BP). Affinity studies using chimeras containing C4BP beta-chain short consensus repeats. J Biol Chem. 1999;274:15144-50.

42. Ploos van Amstel HK, Reitsma PH, Bertina RM. The human protein S locus: identification of the PS alpha gene as a site of liver protein S messenger RNA synthesis. Biochem Biophys Res Commun. 1988;157:1033-8.

43. Schmidel DK, Tatro AV, Phelps LG, Tomczak JA, Long GL. Organization of the human protein S genes. Biochemistry. 1990;29:7845-52.

44. Rosing J, Hoekema L, Nicolaes GA, Thomassen MC, Hemker HC, Varadi K, Schwarz HP, Tans G. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor VaR506Q by activated protein C. J Biol Chem. 1995;270:27852-8.

45. Egan JO, Kalafatis M, Mann KG. The effect of Arg306-->Ala and Arg506-->Gln substitutions in the inactivation of recombinant human factor Va by activated protein C and protein S. Protein Sci. 1997;6:2016-27.

46. Yegneswaran S, Wood GM, Esmon CT, Johnson AE. Protein S alters the active site location of activated protein C above the membrane surface. A fluorescence resonance energy transfer study of topography. J Biol Chem. 1997;272:25013-21.

47. Maurissen LF, Thomassen MC, Nicolaes GA, Dahlbäck B, Tans G, Rosing J, Hackeng TM. Re-evaluation of the role of the protein S-C4b binding protein complex in activated protein C-catalyzed factor Va-inactivation. Blood. 2008;111:3034-41.

48. Shen L, Dahlbäck B. Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. J Biol Chem. 1994;269:18735-8.

49. O'Brien LM, Mastri M, Fay PJ. Regulation of factor VIIIa by human activated protein C and protein S: inactivation of cofactor in the intrinsic factor Xase. Blood. 2000;95:1714-20.

50. Ndonwi M, Tuley EA, Broze GJ Jr. The Kunitz-3 domain of TFPI-alpha is required for protein S-dependent enhancement of factor Xa inhibition. Blood. 2010;116:1344-51.

51. de Fouw NJ, Haverkate F, Bertina RM, Koopman J, van Wijngaarden A, van Hinsbergh VW. The cofactor role of protein S in the acceleration of whole blood clot lysis by activated protein C in vitro. Blood. 1986;67:1189-92.

52. Mosnier LO, Meijers JC, Bouma BN. The role of protein S in the activation of thrombin activatable fibrinolysis inhibitor (TAFI) and regulation of fibrinolysis. Thromb Haemost. 2001;86:1040-6.

53. Bertina RM. Proposal for the nomenclature of protein S deficiency. XXXVIII Annual meeting Scientific and Standardization Committee of the ISTH, Munchen, Germany, 1992.

54. Said JM, Ignjatovic V, Monagle PT, Walker SP, Higgins JR, Brennecke SP. Altered reference ranges for protein C and protein S during early pregnancy: implications for the diagnosis of protein C and protein S deficiency during pregnancy. Thromb Haemost. 2010;103:984-8.

55. Vigano-D'Angelo S, D'Angelo A, Kaufman CE, Jr., Sholer C, Esmon CT, Comp PC. Protein S deficiency occurs in the nephrotic syndrome. Ann Intern Med. 1987;107:42-7.

56. Brouwer JL, Bijl M, Veeger NJ, Kluin-Nelemans HC, van der Meer J. The contribution of inherited and acquired thrombophilic defects, alone or combined with antiphospholipid antibodies, to venous and arterial thromboembolism in patients with systemic lupus erythematosus. Blood. 2004;104:143-8.

57. Lijfering WM, Sprenger HG, Georg RR, van der Meulen PA, van der Meer J. Relationship between progression to AIDS and thrombophilic abnormalities in HIV infection. Clin Chem. 2008;54:1226-33.

58. Henkens CMA, Bom VJJ, van der Schaaf W, Pelsma PM, Sibinga CTS, de Kam PJ, van der Meer J. Plasma-Levels of protein-S, protein-C, and factor-X - effects of sex, hormonal state and age. Thromb Haemost. 1995;74:1271-5.

59. Boerger LM, Morris PC, Thurnau GR, Esmon CT, Comp PC. Oral contraceptives and gender affect protein S status. Blood. 1987;69:692-4.

60. Favaloro EJ, Soltani S, McDonald J, Grezchnik E, Easton L. Laboratory identification of familial thrombophilia: do the pitfalls exceed the

benefits? A reassessment of ABO-blood group, gender, age, and other laboratory parameters on the potential influence on a diagnosis of protein C, protein S, and antithrombin deficiency and the potential high risk of a false positive diagnosis. Lab Hematol. 2005;11:174-84.

61. Goodwin AJ, Rosendaal FR, Kottke-Marchant K, Bovill EG. A review of the technical, diagnostic, and epidemiologic considerations for protein S assays. Arch Pathol Lab Med. 2002;126:1349-66.

62. Mahasandana C, Suvatte V, Marlar RA, Manco-Johnson MJ, Jacobson LJ, Hathaway WE. Neonatal purpura fulminans associated with homozygous protein S deficiency. Lancet. 1990;335:61-2.

63. Gandrille S, Borgel D, Sala N, Espinosa-Parrilla Y, Simmonds R, Rezende S, Lind B, Mannhalter C, Pabinger I, Reitsma PH, Formstone C, Cooper DN, Saito H,Suzuki K, Bernardi F, Aiach M; Plasma Coagulation Inhibitors Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. Protein S deficiency: a database of mutations--summary of the first update. Thromb Haemost. 2000;84:918.

64. Bertina RM, Ploos van Amstel HK, van Wijngaarden A, Coenen J, Leemhuis MP, Deutz-Terlouw PP, van der Linden IK, Reitsma PH. Heerlen polymorphism of protein S, an immunologic polymorphism due to dimorphism of residue 460. Blood. 1990;76:538-48.

65. Duchemin J, Gandrille S, Borgel D, Feurgard P, Alhenc-Gelas M, Matheron C, et al. The Ser 460 to Pro substitution of the protein S alpha (PROS1) gene is a frequent mutation associated with free protein S (type IIa) deficiency. Blood. 1995;86:3436-43.

66. Espinosa-Parrilla Y, Navarro G, Morell M, Abella E, Estivill X, Sala N. Homozygosity for the protein S Heerlen allele is associated with type I PS deficiency in a thrombophilic pedigree with multiple risk factors. Thromb Haemost. 2000;83:102-6.

67. Denis CV, Roberts SJ, Hackeng TM, Lenting PJ. In vivo clearance of human protein S in a mouse model: influence of C4b-binding protein and the Heerlen polymorphism. Arterioscler Thromb Vasc Biol. 2005;25:2209-15.

68. Koenen RR, Gomes L, Tans G, Rosing J, Hackeng TM. The Ser460Pro mutation in recombinant protein S Heerlen does not affect its APC-cofactor and APC-independent anticoagulant activities. Thromb Haemost. 2004;91:1105-14.

69. Dykes AC, Walker ID, McMahon AD, Islam SIAM, Tait RC. A study of Protein S antigen levels in 3788 healthy volunteers: influence of age, sex and hormone use, and estimate for prevalence of deficiency state. Br J Haematol. 2001;113:636-41.

70. Seligsohn U, Lubetsky A. Genetic susceptibility to venous thrombosis. N Engl J Med. 2001;344:1222-31.

71. Libourel EJ, Bank I, Veeger NJ, Hamulyàk K, Middeldorp S, Prins MH, Büller HR, van der Meer J. Protein S type III deficiency is no risk factor for venous and arterial thromboembolism in 168 thrombophilic families: a retrospective study. Blood Coagul Fibrinolysis. 2005;16:135-40.

72. Brouwer JL, Veeger NJ, van der Schaaf W, Kluin-Nelemans HC, van der Meer J. Difference in absolute risk of venous and arterial thrombosis between familial protein S deficiency type I and type III. Results from a family cohort study to assess the clinical impact of a laboratory test-based classification. Br J Haematol. 2005;128:703-10.

73. Romano N, Prosperi V, Basili G, Lorenzetti L, Gentile V, Luceretti R, Biondi G, Goletti O. Acute thrombosis of the superior mesenteric artery in a 39-year-old woman with protein-S deficiency: a case report. J Med Case Reports. 2011;5:17.

74. Archer KA, Lembo T Jr, Haber JA. Protein S deficiency and lower-extremity arterial thrombosis: complicating a common presentation. J Am Podiatr Med Assoc. 2007;97:151-5.

75. Zimmerman AA, Watson RS, Williams JK. Protein S deficiency presenting as an acute postoperative arterial thrombosis in a four-year-old child. Anesth Analg. 1999;88:535-7.

76. Green D, Otoya J, Oriba H, Rovner R. Protein S deficiency in middle-aged women with stroke. Neurology. 1992;42:1029-33.

Chapter 2

Clinical relevance of decreased free protein S levels. Results from a retrospective family cohort study involving 1143 relatives

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SUMMARY

Conflicting data have been reported on the risk for venous thrombosis in subjects with low free protein S levels.

We performed a post-hoc analysis in a single-center retrospective thrombophilic family cohort, to define the optimal free protein S level that can identify subjects at risk for venous thrombosis. Relatives (1143) were analyzed. Relatives with venous thrombosis (mean age 39 years) had lower free protein S levels than relatives without venous thrombosis (P<0.001), which was most pronounced in the lowest quartile. Only relatives with free protein S levels less than the 5th percentile (< 41 IU/dL) or less than the 2.5th percentile (< 33 IU/dL) were at higher risk of first venous thrombosis compared with the upper quartile (> 91 IU/dL); annual incidence 1.20% (95% confidence interval [CI], 0.72-1.87) and 1.81% (95% CI, 1.01-2.99), respectively; adjusted hazard ratios 5.6, (95% CI, 2.7-11.5) and 11.3 (95% CI, 5.4-23.6). Recurrence rates were 12.12% (95 CI, 5.23-23.88) and 12.73% (95% CI, 5.12-26.22) per year; adjusted hazard ratios were 3.0 (95% CI, 1.03-8.5) and 3.4 (95% CI, 1.1-10.3).

In conclusion, free protein S level can identify young subjects at risk for venous thrombosis in thrombophilic families, although the cut-off level lies far below the normal range in healthy volunteers.

INTRODUCTION

Protein S is a vitamin K-dependent plasma glycoprotein that functions as a nonenzymatic cofactor of activated protein C in the inactivation of the procoagulant factors Va and VIIIa, and plays an important role in regulating thrombin generation (1). Approximately 60% of total protein S is bound to complement component C4-binding protein (2). Until recently, it was believed that only free protein S has activated protein C cofactor activity. However, new evidence suggests that both bound and free protein S are cofactors for activated protein C (3). Three subtypes of protein S deficiency are recognized: type I, with decreased levels of both total and free protein S antigen (ie, a quantitative defect); type II, with total and free protein S antigen levels within their normal ranges, but decreased protein S activity (ie, a qualitative defect); and type III, with decreased free protein S and normal total protein S antigen levels (4). Whereas type I deficiency is an established risk factor for venous thrombosis (5, 6), conflicting data have been reported on the risk of thrombosis associated with type III deficiency (6-14). A difference in risk between subjects with type I and type III deficiency, together with variation in the studied populations may explain this discrepancy. Another explanation could be that the cut-off level for protein S deficiency type III (i.e., the lower limit of the normal range; in our laboratory < 65 IU/dL) is too high to identify subjects at risk for venous thrombosis (11). Moreover, studies that did not show an increased risk of venous thrombosis in association with free protein S deficiency may have been confounded by the inclusion of subjects with mild decrements in free protein S levels. The diagnostic criteria for type III protein S deficiency therefore likely need to be reconsidered. We performed a retrospective study in a large series of families to assess the absolute risk of first venous thrombosis and its recurrence for different free protein S levels, to define an optimal cut-off level of free protein S to identify subjects at risk for venous thrombosis.

MATERIAL AND METHODS

Data retrieval

We pooled data of individual subjects from 5 large retrospective family cohort studies with various thrombophilic index defects, which have been described previously (5, 11, 15-17). These studies had the same design and were performed by 3 university hospitals. The first 2 studies were single centered and performed in our hospital. It comprised first-degree relatives (i.e., offspring, siblings, and/or parents) of consecutive patients (probands) with documented venous thrombosis and established hereditary deficiencies of either antithrombin, protein C, or protein S (5, 11). As the number of antithrombin deficient probands was small, second-degree relatives (i.e., grandparents and/or blood related uncles or aunts) with a deficient parent were also identified. They were enrolled between April 1999 and July 2004. Three studies were multicenter studies of first-degree relatives of consecutive patients with venous thrombosis or premature atherosclerosis (< 50 years of age) and the presence of either the prothrombin G20210A mutation, high levels of factor VIII at repeated measurements, or hyperhomocysteinemia (15-17). Enrollment started in May 1998 and was completed in July 2004. Probands were tested on multiple thrombophilic defects in all 5 studies. When multiple defects were demonstrated, probands were randomly assigned to one of the 5 study cohorts (5, 11, 15-17). As laboratory tests were locally performed, using different free protein S assays within the 3 centers and different calibration lines, we chose to only include the data obtained from subjects in our center. Approval was obtained from the institutional review board of University Medical Center Groningen.

Subjects

All relatives, identified by pedigree analysis, were 15 years of age or older and were contacted through the probands. Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. Physicians at our thrombosis outpatient clinic collected detailed

information about previous episodes of venous thrombosis, exposure to exogenous risk factors for venous thrombosis, and anticoagulant treatment using a validated questionnaire (18), and by reviewing medical records. Clinical data were collected before laboratory testing. Relatives were tested for hereditary deficiencies of antithrombin, protein C and protein S type I, factor V Leiden, prothrombin G20210A, and high levels of factor VIII, regardless of their index defects. In addition, levels of free protein S were measured in most, but not all, relatives due to shortage of stored plasma. To avoid bias, relatives with protein S deficiency type I as well as all probands were excluded from the analyses.

Laboratory studies

Activity of antithrombin (Chromogenix, Mölndal, Sweden) and protein C (Behring, Marburg, Germany) were measured by chromogenic substrate assays, protein C, and protein S antigen levels by enzyme-linked immunosorbent assay (ELISA; DAKO, Glostrup, Denmark). Antithrombin deficiency was defined by decreased levels of antithrombin activity (< 70 IU/dL), protein C deficiency by decreased levels of either protein C antigen (< 65 IU/dL) and/or activity (< 65 IU/dL), and protein S deficiency type I by decreased total (< 65 IU/dL) and free protein S antigen levels (< 65 IU/dL), corresponding with plasma levels below the lower limit of their normal ranges. Strict criteria for inheritance of deficiencies were used (5). Free protein S antigen levels were measured after precipitation of protein S complexed with C4-binding protein with polyethylene glycol (19). Normal values were obtained from a group of healthy volunteers, balanced for gender, without a history of venous thrombosis. The female volunteers did not use oral contraceptives and were not pregnant. Factor V Leiden and prothrombin G20210A were demonstrated by polymerase chain reactions (PCRs) (20, 21). Factor VIII levels were measured by one-stage clotting assays and were considered increased at levels above 150 IU/dL (16). If relatives were on treatment with acenocoumarol, a short-acting vitamin K antagonist, blood samples were taken after treatment had been interrupted for at least 2 weeks if possible; meanwhile, nadroparin was given subcutaneously.

Definitions

The first episode of venous thrombosis was considered established if deep vein thrombosis was confirmed by compression ultrasound or venography, and pulmonary embolism by ventilation/perfusion lung scanning, spiral computed tomography (CT) scanning or pulmonary angiography, or when the patient had received full-dose heparin and a vitamin K antagonist for at least 3 months without objective testing at a time when these techniques were not yet available. Superficial phlebitis was not classified as a thrombotic event. If recurrent deep vein thrombosis at the same site was suspected, but objective tests were not conclusive, it was considered ascertained if the patient revealed pronounced signs and symptoms of recurrence without preceding postthrombotic syndrome, or when pulmonary embolism was objectively demonstrated. Venous thrombosis was defined provoked if it occurred within 3 months after exposure to exogenous risk factors including surgery, trauma, immobilization for more than 7 days, pregnancy, postdelivery period, the use of oral contraceptives or hormonal replacement therapy, or malignancy. In the absence of these risk factors, venous thrombosis was classified idiopathic.

Statistical analysis

Cumulative distribution functions were constructed to visualize a possible relationship between free protein S levels and the occurrence of venous thrombosis. To find an optimal cut-off value of free protein S or a possible dose response relationship, we stratified free protein S levels into quartiles and levels lower than the 5^{th} and 2.5^{th} percentiles to calculate the absolute risk of both first and recurrent venous thrombosis in these subgroups. Observation time was defined as the period from the age of 15 years until the first thrombotic episode or the end of study. The absolute risk of recurrent venous thrombosis was calculated over the period from the end of anticoagulant treatment after the first episode of venous thrombosis until either the date of first recurrence or the end of study. Incidences and 95% confidence intervals (95% CIs) were calculated under the Poisson distribution assumption. Freedom of recurrent venous thrombosis was

analyzed by the Kaplan-Meier method. Hazard ratios were calculated using a multivariable Cox regression model. As our study cohort consisted of subjects from thrombophilic families and were therefore prone to have multiple thrombophilic defects (5, 22), we first adjusted for these defects with stepwise Cox regression, including antithrombin or protein C deficiency, factor V Leiden, prothrombin G20210A, and high factor VIII levels. To account for the nonrandomness of the relatives analyzed, outcome rates were also adjusted for clustering of events within families with Cox regression analysis and the robust sandwich method (in SAS 9.1). In this analysis, all relatives were identified by a family number, which included a code for the type of thrombophilic defect. Hazard ratios were also adjusted for age and sex. Continuous variables were expressed as mean values and standard deviations; categorical data as counts and percentages. Differences between groups were evaluated by the Student *t* test or Mann-Whitney U test, depending on the normality of data for continuous variables and by Fisher exact test for categorical variables. A 2-tailed *P*-value of less than 0.05 indicated statistical significance. Statistical analyses were performed using SAS software, version 9.1 (SAS Institute, Cary, NC).

RESULTS

Our study cohort comprised 2451 relatives aged 15 years or older, of 380 probands (Figure 1). Of relatives, 518 (21%) had died before the start of the study. Another 465 relatives did not participate because of various reasons, including refusal, inability to give informed consent, or residence outside The Netherlands (exclusion rate 24%). A total of 94 relatives were excluded because of protein S deficiency type I, and 231 relatives could not be evaluated because of missing laboratory data. Forty-seven percent were male (Table 1). Mean plus or minus standard deviation (SD) age at enrollment was 45 plus or minus 17 years. Mean observation period was 29 plus or minus 16 years. Mean free protein S level was 80 plus or minus 20 IU/dL; 83 plus or minus 26 IU/dL in men and 75 plus or minus 24 IU/dL in women ($P<0.001$).

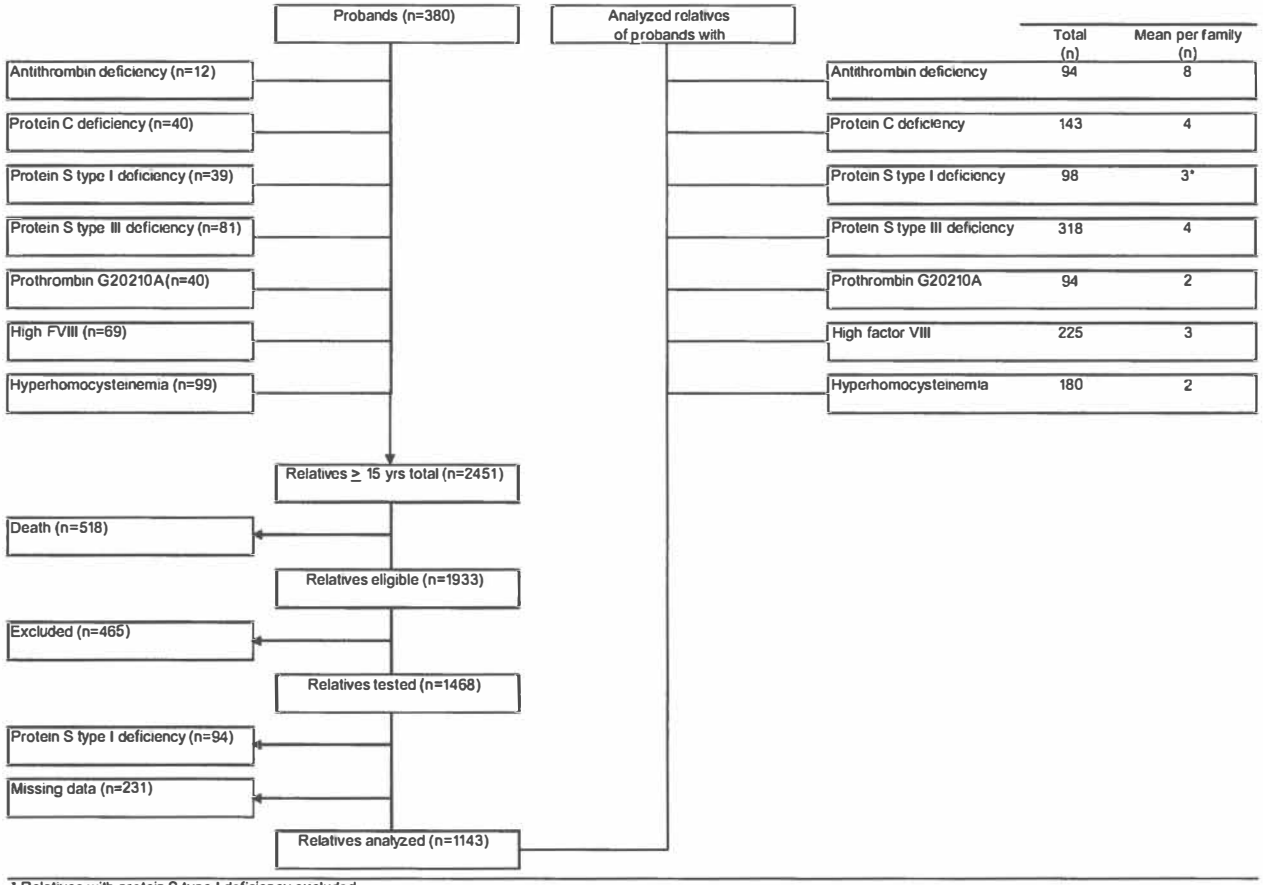

Figure 1. Flow diagram of the family cohort.

Table 1. Characteristics of 1143 relatives of probands with a thrombophilic defect

Male	532	(47)
Age at enrolment	45	(17)
Free protein S levels IU/dL	80	(22)
In males	83	(25)
In females	75	(21)
First venous thrombosis	96	(8)
Age at onset	39	(15)
Classification		
Idiopathic	34	(35)
Provoked	62	(65)
Surgery, trauma, immobilization	26	
Oral contraception/ hormonal replacement therapy	18	
Pregnancy, puerperium	17	
Malignancy	1	
Concomitant thrombophilic defects		
Antithrombin deficiency	54	(5)
Protein C deficiency	69	(6)
Factor V Leiden	159	(14)
Prothrombin G20210A	97	(8)
Factor VIII > 150 IU/dL	443	(39)

Continuous variables denoted as mean (standard deviation)
categorical variables as number (%).

Free protein S levels less than 50 IU/dL were equally distributed in men and women (Figure 2). Venous thrombosis had occurred in 96 relatives (8%). Mean age at onset of the first episode of venous thrombosis was 39 plus or minus 15 years. Median calendar date of onset of first venous thrombosis was August 1991 (range, January 1955-June 2004).

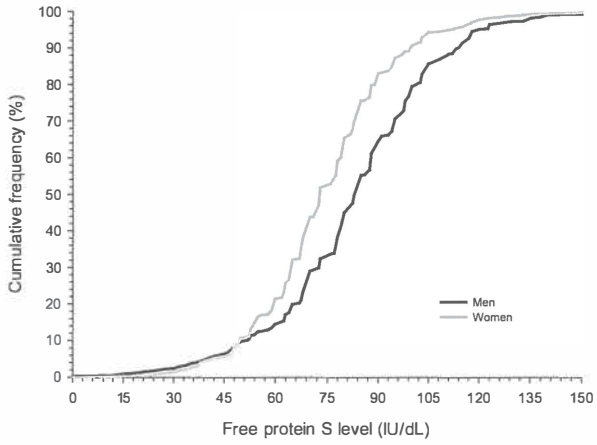

Figure 2. Cumulative distribution function of free protein S levels in male and female relatives of probands with a thrombophilic defect.

A histogram showed a normal distribution of free protein S levels in the study cohort, with a left shift from the normal mean (i.e., 100 IU/dL; Figure 3).

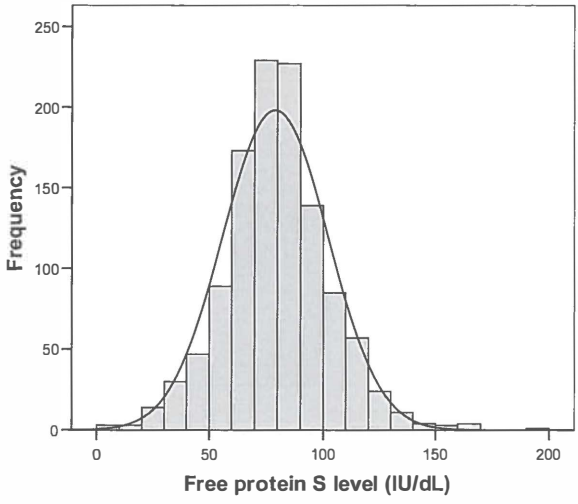

Figure 3. Distribution of free protein S levels in relatives of probands with a thrombophilic defect.

Relatives with venous thrombotic events had lower free protein S levels than relatives without venous thrombosis (P<0.001), which was most pronounced in the lowest quartile (Figure 4).

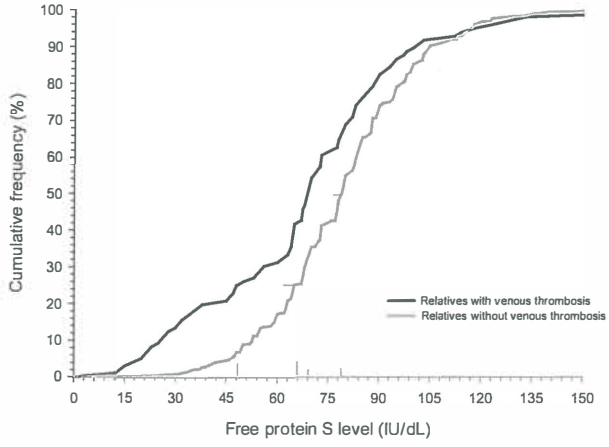

Figure 4. Cumulative distribution function of free protein S levels in relatives of probands with a thrombophilic defect.
Gray dashed, dashed dotted and dotted lines display free protein S level for relatives with and without venous thrombosis at cumulative frequency of 25%, 50% and 75%, respectively.

Annual incidences of first venous thrombosis in relatives with free protein S levels more than the 75th percentile, between the 50th and 75th percentile, the 25th and 50th percentile, the 5th and 25th percentile, less than the 5th percentile, and less than the 2.5th percentile were 0.20%, 0.21%, 0.31%, 0.26%, 1.20%, and 1.81%, respectively (Table 2). Concomitance of thrombophilic defects were observed in 87% to 100% of relatives with an event within the 6 analyzed groups. Only relatives with free protein S levels below the 5th percentile (< 41 IU/dL) or below the 2.5th percentile (< 33 IU/dL) were at higher risk of first venous thrombosis compared with relatives with free protein S levels in the upper quartile; crude hazard ratio 5.7 (95% CI, 2.9–10.1) and 10.8 (95% CI, 5.2–22.1), respectively. Adjusted for age, sex, and clustering of events in families, hazard ratios were 5.6 (95% CI,

2.7–11.5) and 11.3 (95% CI, 5.4–23.6), respectively. To account for possible misclassification, as not all events were confirmed by objective techniques because these were not available at the time of onset, we excluded relatives with clinically diagnosed first venous thrombosis (n = 35) from our cohort and repeated the analysis that adjusted for age, sex, and clustering within families. Adjusted hazard ratios for first venous thrombosis in relatives with free protein S levels, between the 50th and 75th percentile, the 25th and 50th percentile, the 5th and 25th percentile, less than the 5th percentile, and less than the 2.5th percentile compared with the upper quartile were 0.8 (95% CI, 0.3–2.1), 1.4 (95% CI, 0.6–3.1), 0.9 (95% CI, 0.3–2.5), 5.3 (95% CI, 2.1–13.6), and 11.6 (95% CI, 4.3–31.2). Cumulative recurrence rates in relatives (not on continuing anticoagulant treatment) with free protein S levels in the aforementioned subgroups were 4.13%, 2.07%, 1.66%, 2.19%, 12.12%, and 12.73% (Table 3), respectively. Relatives with free protein S levels lower than the 5th or 2.5th percentile had an adjusted 3.0-fold (95% CI, 1.03–8.5) and 3.4-fold (95% CI, 1.1–10.3) higher risk of recurrence compared with relatives with free protein S levels in the upper quartile.

Table 2. Risk of first venous thrombosis risk for strata of free protein S levels

Range	Observation years (relatives)	Relatives with event	Annual incidence, % (95% CI)	Crude HR (95% CI)	Adjusted HR[2] (95% CI)	Adjusted HR[1] (95% CI)	Adjusted HR (95% CI) adjusted for Antithrombin deficiency	Protein C deficiency	Factor V Leiden	Prothrombin G20210A	High factor VIII
> 75th percentile (> 91 IU/dL)	8586 (n = 291)	17	0.20 (0.11-0.32)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
50th-75th percentile (79-90 IU/dL)	8044 (n = 285)	17	0.21 (0.12-0.34)	1.0 (0.5-2.1)	0.9 (0.4-2.0)	1.0 (0.4-2.3)	1.2 (0.6-2.4)	1.0 (0.5-2.0)	1.1 (0.6-2.2)	1.1 (0.6-2.2)	1.0 (0.5-2.1)
25th-50th percentile (65-78 IU/dL)	9168 (n = 308)	28	0.31 (0.20-0.44)	1.5 (0.8-2.8)	1.4 (0.7-2.8)	1.2 (0.5-2.8)	1.7 (0.9-3.2)	1.5 (0.8-2.8)	1.6 (0.8-2.9)	1.6 (0.9-3.0)	1.4 (0.8-2.6)
5th-25th percentile (41-64 IU/dL)	5856 (n = 205)	15	0.26 (0.14-0.42)	1.2 (0.6-2.4)	1.1 (0.5-2.6)	1.1 (0.5-2.5)	1.6 (0.8-3.3)	1.3 (0.6-2.6)	1.3 (0.6-2.6)	1.3 (0.6-2.6)	1.2 (0.6-2.4)
< 5th percentile (< 41 IU/dL)	1589 (n = 54)	19	1.20 (0.72-1.87)	5.7 (2.9-10.9)	5.6 (2.7-11.5)	5.9 (2.9-12.0)	5.9 (3.0-11.5)	3.9 (1.9-7.8)	5.7 (2.9-11.1)	5.9 (3.0-11.6)	5.7 (3.0-11.1)
< 2.5th percentile (< 33 IU/dL)	828 (n = 28)	15	1.81 (1.01-2.99)	10.8 (5.2-22.1)	11.3 (5.4-23.6)	11.8 (5.5-25.6)	9.3 (4.5-19.2)	7.6 (3.5-16.2)	11.1 (5.2-23.5)	11.3 (5.4-23.5)	10.9 (5.3-22.5)

- Number of relatives with protein S type III deficiency (free protein S levels < 65 IU/dL) was 259 (23%).
- HR denotes hazard ratio.

Table 3. Risk of recurrent venous thrombosis for strata of free protein S levels

Range	Observation years (relatives)	Relatives with event	Annual incidence, % (95% CI)	Crude HR (95% CI)	Adjusted HR* (95% CI)	Adjusted HR† (95% CI)	Adjusted HR (95% CI) adjusted for Antithrombin deficiency	Protein C deficiency	Factor V Leiden	Prothrombin G20210A	High factor VIII
> 75th percentile (> 91 IU/dL)	121 (n = 12)	5	4.13 (1.34-9.64)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
50th-75th percentile (79-90 IU/dL)	145(n = 14)	3	2.07 (0.43-6.05)	0.5 (0.1-2.3)	0.6 (0.2-2.3)	0.7 (0.2-3.4)	1.2 (0.2-6.5)	0.6 (0.1-2.3)	0.5 (0.1-2.1)	0.4 (0.1-2.3)	0.6 (0.1-2.4)
25th-50th percentile (65-78 IU/dL)	241(n = 25)	4	1.66 (0.45-4.25)	0.5 (0.1-1.7)	0.5 (0.2-1.9)	0.9 (0.2-3.4)	0.9 (0.2-4.0)	0.4 (0.1-1.6)	0.4 (0.1-1.5)	0.4 (0.1-1.3)	0.7 (0.1-3.5)
5th-25th percentile (41-64 IU/dL)	228(n = 14)	5	2.19 (0.71-5.12)	0.6 (0.2-2.3)	0.7 (0.2-2.6)	0.7 (0.2-2.7)	0.6 (0.6-2.1)	0.7 (0.2-2.4)	0.5 (0.1-1.9)	0.8 (0.2-2.7)	0.7 (0.2-2.4)
< 5th percentile (< 41 IU/dL)	66(n = 11)	8	12.12 (5.23-23.88)	2.8 (0.9-8.6)	3.0 (1.03-8.5)	3.1 (0.9-10.8)	4.3 (1.2-15.0)	2.7 (0.9-8.3)	2.5 (0.8-8.0)	2.2 (0.7-7.4)	4.4 (1.2-15.9)
< 2.5th percentile (< 33 IU/dL)	55(n = 10)	7	12.73 (5.12-26.22)	3.1 (0.95-10.0)	3.4 (1.1-10.3)	3.1 (0.9-10.8)	4.6 (1.3-16.7)	4.7 (1.2-17.6)	2.9 (0.8-10.3)	2.5 (0.7-9.1)	4.6 (1.2-16.9)

- HR denotes hazard ratio.
- * Adjusted for age, sex, and clustering of events within families.
- † Excluding women who used estrogens at time of venous thrombosis or at enrollment and adjusted for age, sex, and clustering of events within families.

DISCUSSION

Most clinical studies on protein S type III deficiency refer to laboratory reference values obtained from healthy volunteers (which in our laboratory is < 65 IU/dL) (6-14). Our study showed that this cut-off level is too high to identify subjects at risk for venous thrombosis due to protein S type III deficiency. Rather, we identified a threshold level, both for first venous thrombosis and its recurrence, at free protein S levels lower than 41 IU/dL (5^{th} percentile). Using this cut-off point increased the absolute risk of first venous thrombosis in our study population from 0.20% per year to 1.20% per year and for recurrence from 4.13% per year to 12.12% per year. These risks are high compared with the general population, with an annual incidence of first venous thrombosis of 0.1% to 0.3% and annual recurrence rate of 3% to 5% (23-26). It should be noted, however, that relatives with first venous thrombosis were relatively young (mean age 39 years vs 62 years in the general population) (27), which emphasizes that thrombophilic defects are risk factors for venous thrombosis at young age (22). It also explains why first episodes of venous thrombosis associated with malignancy were scarce in our study. Although numbers were small, it is tempting to speculate that subjects with venous thrombosis and free protein S levels in the lower 5th percentile could benefit from prolonged anticoagulant treatment, given the high rate of recurrence. Free protein S levels in our study (mean 80 IU/dL) showed a left shift compared with the normal population (mean 100 IU/dL), which is likely a result of including thrombophilic families, although we did not exclude from analysis women who were on oral contraceptives. However, more than 60% of patients with venous thrombosis have at least one thrombophilic defect (28), whereas mostly relatives of patients with thrombophilic defects are seeking advice concerning screening and risk management (29), whereas in The Netherlands, more than 40% of fertile women use oral contraceptives (30). Therefore, the results of this study probably concern young subjects that are screened in daily practice. It is noteworthy that low free protein S levels are seen in patients with systemic

lupus erythematosus (31), the nephrotic syndrome (32), or HIV infection (33), conditions that are all associated with a higher risk for venous thrombosis. It is not likely that our results were influenced by these conditions according to the rare disease assumption. Nevertheless, low free protein S levels probably involve both genetic and acquired (transient) factors (34). As genotype-phenotype associations on free protein S levels was not performed in all our subjects, this may be a limitation since it would have enabled us a more accurate classification. Some methodologic aspects of our study warrant comment. First, as events were not always confirmed by objective techniques, our absolute risk estimates may have been overestimated by misclassification. We assume, however, that misclassification would occur equally over all the free protein S strata, and therefore would not change our relative risk estimates. Moreover, relative risk estimates did not change when we excluded relatives with clinically diagnosed first venous thrombosis. Second, referral bias may have been introduced by the university hospital setting. However, this was probably reduced by testing consecutive patients with thrombosis. Third, oral contraceptive use and hormonal replacement therapy decrease free protein S levels (35) and are known risk factors for venous thrombosis as well (36). Adjustment for these possible confounders was difficult as we only had information on used oral contraceptives or hormonal replacement therapy at time of venous thrombosis or at time of blood sampling. When low free protein S levels are regarded as a risk factor for venous thrombosis, adjustment for estrogen use with regression analysis is not appropriate, considering that estrogens may be part of the causal pathway. Therefore, the data were reanalyzed, excluding women who were on oral contraceptives or hormonal replacement therapy at time of enrollment or at onset of venous thrombosis. This approach did not change our main outcomes. Fourth, as expected, because we enrolled relatives of probands with a thrombophilic defect, concomitance of thrombophilic defects in relatives with venous thrombosis was observed more frequently than in the normal population (i.e., approximately 60%, not including free protein S deficiency) (28). However, low free protein S levels remained a risk factor for venous thrombosis after we adjusted for concomitance of

separate thrombophilic defects and for clustering of events within families. Fifth, by pooling data from several studies, a very large sample size was obtained, which is a strength of the study. Including more than 1000 subjects allowed us to not only establish a relationship between decreased free protein S and the risk of venous thrombosis, but to also be able to stratify this risk according to the level of free protein S. This pooled analysis only looked at subjects who were tested at a single hospital, thereby allowing a single reference range to be established and eliminating inter-laboratory assay variability. Finally, although we obtained a study population of a large size by pooling different family studies, not all relatives were tested for free protein S levels. Because we did a post-hoc analysis of data, we are not able to retrieve this missing data. Bias, however, seems unlikely as free protein S levels were normally distributed in our study cohort.

In conclusion, our study demonstrates that decreased free protein S levels are a risk factor for venous thrombosis in subjects at a young age in thrombophilic families. However, the laboratory cut-off level lies far below the lower limit of the normal range in healthy volunteers.

REFERENCES

1. Simioni P, Tormene D, Spiezia L, Tognin G, Rossetto V, Radu C, Prandoni P. Inherited thrombophilia and venous thromboembolism. Semin Thromb Hemost. 2006;32:700-8.

2. Dahlbäck B. The tale of protein S and C4b-binding protein, a story of affection. Thromb Haemost. 2007;98:90-6.

3. Maurissen LF, Thomassen MC, Nicolaes GA, Dahlbäck B, Tans G, Rosing J, Hackeng TM. Re-evaluation of the role of the protein S-C4b binding protein complex in activated protein C-catalyzed factor Va-inactivation. Blood. 2008;111:3034-41.

4. De Stefano V, Finazzi G, Mannucci PM. Inherited thrombophilia: pathogenesis, clinical syndromes, and management. Blood. 1996;87:3531-44.

5. Brouwer JL, Veeger NJ, Kluin-Nelemans HC, van der Meer J. The pathogenesis of venous thromboembolism: evidence for multiple interrelated causes. Ann Intern Med. 2006;145:807-15.

6. Martinelli I, Mannucci PM, De Stefano V, Taioli E, Rossi V, Crosti F, Paciaroni K, Leone G, Faioni EM. Different risks of thrombosis in four coagulation defects associated with inherited thrombophilia: a study of 150 families. Blood. 1998;92:2353-8.

7. Makris M, Leach M, Beauchamp NJ, Daly ME, Cooper PC, Hampton KK, Bayliss P, Peake IR, Miller GJ, Preston FE. Genetic analysis, phenotypic diagnosis, and risk of venous thrombosis in families with inherited deficiencies of protein S. Blood. 2000;95:1935-41.

8. Simmonds RE, Zöller B, Ireland H, Thompson E, de Frutos PG, Dahlbäck B, Lane DA. Genetic and phenotypic analysis of a large (122-member) protein S-deficient kindred provides an explanation for the familial coexistence of type I and type III plasma phenotypes. Blood. 1997;89:4364-70.

9. Borgel D, Duchemin J, Alhenc-Gelas M, Matheron C, Aiach M, Gandrille S. Molecular basis for protein S hereditary deficiency: genetic defects observed in 118 patients with type I and type IIa deficiencies. The French Network on Molecular Abnormalities Responsible for Protein C and Protein S Deficiencies. J Lab Clin Med. 1996;128:218-27.

10. Faioni EM, Valsecchi C, Palla A, Taioli E, Razzari C, Mannucci PM. Free protein S deficiency is a risk factor for venous thrombosis. Thromb Haemost. 1997;78:1343-6.

11. Brouwer JL, Veeger NJ, van der Schaaf W, Kluin-Nelemans HC, van der Meer J. Difference in absolute risk of venous and arterial thrombosis between familial protein S deficiency type I and type III. Results from a family cohort study to assess the clinical impact of a laboratory test-based classification. Br J Haematol. 2005;128:703-10.

12. Bertina RM, Ploos van Amstel HK, van Wijngaarden A, Coenen J, Leemhuis MP, Deutz-Terlouw PP, van der Linden IK, Reitsma PH. Heerlen polymorphism of protein S, an immunologic polymorphism due to dimorphism of residue 460. Blood. 1990;76:538-48.

13. Duchemin J, Gandrille S, Borgel D, Feurgard P, Alhenc-Gelas M, Matheron C, Dreyfus M, Dupuy E, Juhan-Vague I, Aiach M. The Ser 460 to Pro substitution of the protein S alpha (PROS1) gene is a frequent mutation associated with free protein S (type IIa) deficiency. Blood. 1995;86:3436-43.

14. Koster T, Rosendaal FR, Briët E, van der Meer FJ, Colly LP, Trienekens PH, Poort SR, Reitsma PH, Vandenbroucke JP. Protein C deficiency in a controlled series of unselected outpatients: an infrequent but clear risk factor for venous thrombosis (Leiden Thrombophilia Study). Blood. 1995;85:2756-61.

15. Bank I, Libourel EJ, Middeldorp S, Van Pampus EC, Koopman MM, Hamulyák K, Prins MH, van der Meer J, Büller HR. Prothrombin 20210A

mutation: a mild risk factor for venous thromboembolism but not for arterial thrombotic disease and pregnancy-related complications in a family study. Arch Intern Med. 2004;164:1932-7.

16. Bank I, Libourel EJ, Middeldorp S, Hamulyák K, van Pampus EC, Koopman MM, Prins MH, van der Meer J, Büller HR. Elevated levels of FVIII:C within families are associated with an increased risk for venous and arterial thrombosis. J Thromb Haemost. 2005;3:79-84.

17. Lijfering WM, Coppens M, van de Poel MH, Middeldorp S, Hamulyák K, Bank I, Veeger NJ, Prins MH, Büller HR, van der Meer J. The risk of venous and arterial thrombosis in hyperhomocysteinaemia is low and mainly depends on concomitant thrombophilic defects. Thromb Haemost. 2007;98:457-63.

18. Frezzato M, Tosetto A, Rodeghiero F. Validated questionnaire for the identification of previous personal or familial venous thromboembolism. Am J Epidemiol. 1996;143:1257-65.

19. Comp PC, Thurnau GR, Welsh J, Esmon CT. Functional and immunologic protein S levels are decreased during pregnancy. Blood. 1986;68:881-5.

20. Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature. 1994;369:64-7.

21. Danneberg J, Abbes AP, Bruggeman BJ, Engel H, Gerrits J, Martens A. Reliable genotyping of the G-20210-A mutation of coagulation factor II (prothrombin). Clin Chem. 1998;44:349-51.

22. Lijfering WM, Brouwer JL, Veeger NJ, Bank I, Coppens M, Middeldorp S, Hamulyák K, Prins MH, Büller HR, van der Meer J. Selective testing for thrombophilia in patients with first venous thrombosis: results from a retrospective family cohort study on absolute thrombotic risk for currently known thrombophilic defects in 2479 relatives. Blood.

2009;113:5314-22.

23. Heit JA, Mohr DN, Silverstein MD, Petterson TM, O'Fallon WM, Melton LJ 3rd. Predictors of recurrence after deep vein thrombosis and pulmonary embolism: a population-based cohort study. Arch Intern Med. 2000;160:761-8.

24. Hansson PO, Welin L, Tibblin G, Eriksson H. Deep vein thrombosis and pulmonary embolism in the general population. 'The Study of Men Born in 1913'. Arch Intern Med. 1997;157:1665-70.

25. Naess IA, Christiansen SC, Romundstad P, Cannegieter SC, Rosendaal FR, Hammerstrøm J. Incidence and mortality of venous thrombosis: a population-based study. J Thromb Haemost. 2007;5:692-9.

26. Prandoni P, Noventa F, Ghirarduzzi A, Pengo V, Bernardi E, Pesavento R, Iotti M, Tormene D, Simioni P, Pagnan A. The risk of recurrent venous thromboembolism after discontinuing anticoagulation in patients with acute proximal deep vein thrombosis or pulmonary embolism. A prospective cohort study in 1,626 patients. Haematologica. 2007;92:199-205.

27. Heit JA, Silverstein MD, Mohr DN, Petterson TM, Lohse CM, O'Fallon WM, Melton LJ 3rd. The epidemiology of venous thromboembolism in the community. Thromb Haemost. 2001;86:452-63.

28. Rosendaal FR. Venous thrombosis: a multicausal disease. Lancet. 1999;353:1167-73.

29. Martinelli I. Pros and cons of thrombophilia testing: pros. J Thromb Haemost. 2003;1:410-1.

30. Central Bureau of Statistics, The Netherlands. 2008 [Accessed August 11, 2008]. http://www.cbs.nl/nl-NL/menu/themas/gezondheid-welzijn/publicaties/artikelen/archief/2003/2003-1279-wm.htm.

31. Brouwer JL, Bijl M, Veeger NJ, Kluin-Nelemans HC, van der Meer J. The contribution of inherited and acquired thrombophilic defects, alone or combined with antiphospholipid antibodies, to venous and arterial

thromboembolism in patients with systemic lupus erythematosus. Blood. 2004;104:143-8.

32. Vigano-D'Angelo S, D'Angelo A, Kaufman CE Jr, Sholer C, Esmon CT, Comp PC. Protein S deficiency occurs in the nephrotic syndrome. Ann Intern Med. 1987;107:42-7.

33. Lijfering WM, Sprenger HG, Georg RR, van der Meulen PA, van der Meer J. Relationship between progression to AIDS and thrombophilic abnormalities in HIV infection. Clin Chem. 2008;54:1226-33.

34. Ten Kate MK, Platteel M, Mulder R, Terpstra P, Nicolaes GA, Reitsma PH, van der Steege G, van der Meer J. PROS1 analysis in 87 pedigrees with hereditary protein S deficiency demonstrates striking genotype-phenotype associations. Hum Mutat. 2008;29:939-47.

35. Bloemenkamp KW, Rosendaal FR, Helmerhorst FM, Koster T, Bertina RM, Vandenbroucke JP. Hemostatic effects of oral contraceptives in women who developed deep-vein thrombosis while using oral contraceptives. Thromb Haemost. 1998;80:382-7.

36. Vandenbroucke JP, Rosing J, Bloemenkamp KW, Middeldorp S, Helmerhorst FM, Bouma BN, Rosendaal FR. Oral contraceptives and the risk of venous thrombosis. N Engl J Med. 2001;344:1527-35.

Chapter 3

Decreased free protein S levels and venous thrombosis in the acute setting, a case-control study

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Hereditary free protein S deficiency is a well-known risk factor for venous thrombosis (VT) (1, 2). Acquired free protein S deficiency has been reported during pregnancy (3), in the nephrotic syndrome (4), in auto-immune disorders (5), and in infections such as human immunodeficiency virus (6, 7). The mechanism by which free protein S levels are lowered in these diseases is supposed to be by increase of complement component C4-binding protein (C4BP) due to the acute phase. However, this hypothesis is challenged. Whether acquired free protein S deficiency increases risk of VT is unknown. Recently, we found that free protein S levels below 41 IU/dL can identify subjects at risk of VT (8). We performed a case-control study to determine whether free protein S levels below 41 IU/dL are also associated with an increased risk of VT in the acute moment, as well as to assess the effects of preceding episodes of infectious symptoms and increased C-reactive protein (CRP) concentrations on free protein S levels. Finally, we tested the role of C4BP in the lowering of free protein S in these patients.

From April 2008 till January 2010, consecutive patients with suspected deep vein thrombosis of the leg(s) were included in this study, after informed consent was given. Cases were patients with confirmed VT by compression ultra sound (CUS). Controls were patients in whom the disease at (repeated) CUS was ruled out (9). Calf vein thrombosis and thrombophlebitis were not included (9). A standardized questionnaire was used before CUS to collect patients' characteristics, including previous VT, risk factors for VT and symptoms of infection experienced during the preceding month (10). Blood was taken by peripheral vena puncture at time of presentation. CRP levels > 5 mg/L were defined as elevated. C4BP protein levels were assessed by spectrophotometry (590 nm) using the Liatest C4b-BP Latex Immunoassay (Roche Diagnostics, Almere, the Netherlands). The assay was standardized with a home-made normal plasma pool, prepared in our laboratory.

Free protein S levels were measured by enzyme-linked immunosorbent assay, after precipitation of protein S complexed with C4BP with polyethylene glycol (3.75% PEG 6000 purchased from Fluka Chemical (Buchs, Switzerland)) (11).

According to our previous study (8), we categorized free protein S levels in five categories (≥ 91, 79-90, 65-78, 41-64 and below 41 IU/dL). Odds ratios and 95% confidence intervals (95%CI) were calculated as an estimate of the relative risk for VT and were adjusted for age, sex, CRP, infectious symptoms and C4BP as defined above. Data management and statistical analysis were performed using SPSS (version 16, SPSS inc. Chicago, Illinois).

Of 289 eligible consecutive patients, we excluded 27 patients with known confounders from analysis to avoid bias (3 cases and 3 controls were pregnant or in puerperium, 16 cases and 3 controls were taking oral contraceptives, and 1 case and 1 control had a known protein S deficiency type I). Also, in 10 cases and 9 controls free protein S levels were not measured, and 22 cases and 5 controls were taking vitamin K antagonists at blood sampling and consequently excluded. Eventually, 216 subjects (97 cases and 119 controls) were analyzed. Sixty-eight percent of cases and 47% of controls were male. Median age was higher in cases than in controls (median age 61 versus 53 years). Forty-six percent of cases had a known risk factor for VT, compared to 40% of controls. A previous episode of VT was experienced by 31% of cases compared to 44% of controls. One-third of cases reported inflammatory symptoms in the four weeks before presentation compared to almost one quarter of controls. Cases had higher median levels of CRP than controls (median 19 versus 5 mg/ml, $P<0.001$). Median total C4BP levels were 180% (range 44-483%) and 184% (range 42-433%) in cases and controls, respectively. Median total protein S antigen levels were 122 IU/dL (range 77-185 IU/dL) and 112 IU/dL (range 65-172 IU/dL) in cases and controls, respectively. No difference between cases and controls was observed for duration of complaints of VT. Median free protein S levels were 76 IU/dL in cases (range 26-125 IU/dL) and 76 IU/dL in controls (range 35-135 IU/dL). However, more cases than controls had free protein S levels below 41 IU/dL, although not statistically significant (5 (5%) versus 2 (2%), $P=0.25$). Of these, one case and one control had a history of VTE. None had a decreased protein S or protein C level, or recently used VKA. CRP levels were elevated in all seven patients.

Tabel 1. Risk of deep vein thrombosis for strata of free protein S levels

	Cases	Controls	Crude OR	Adjusted OR*	Adjusted OR†	Adjusted OR ¶	Adjusted OR ‖
	n=97	n=119	(95% CI)	(95% CI)	(95% CI)	(95% CI)	(95% CI)
Free protein S ≥91 IU/dL	23	31	Reference	Reference	Reference	Reference	Reference
Free protein S 79-91 IU/dL	22	22	1.3 (0.6-3.0)	1.4 (0.6-3.3)	1.5 (0.6-3.5)	1.6 (0.7-3.7)	1.5 (0.7-3.6)
Free protein S 65-79 IU/dL	23	31	1.0 (0.5-2.1)	1.0 (0.5-2.3)	1.0 (0.5-2.3)	0.8 (0.4-1.9)	1.0 (0.5-2.3)
Free protein S 41-65 IU/dL	24	33	1.0 (0.5-2.1)	1.2 (0.6-2.8)	1.3 (0.6-3.1)	1.0 (0.4-2.4)	1.1 (0.5-2.5)
Free protein S <41 IU/dL	5	2	3.4 (0.6-18.9)	3.6 (0.6-20.8)	3.5 (0.6-20.2)	1.9 (0.3-11.5)	3.9 (0.7-23.1)

* Adjusted for age and sex.

† Adjusted for age, sex and inflammatory symptoms.

¶ Adjusted for age, sex and C-reactive protein.

‖ Adjusted for age, sex and C4BP.

OR denotes odds ratio.

Compared to the highest level of free protein S (≥91 IU/dL), we did not find an increased risk of VT for the next three lower categories (90-41 IU/dL) of free protein S (Table 1). However, patients with free protein S levels below 41 IU/dL seemed to be at higher risk of VT, crude odds ratio 3.4 (95%CI, 0.6-18.9). This risk did not change after adjustment for age, sex and infectious symptoms. However, when infectious symptoms were replaced by CRP in the multivariate analysis, the odds ratio of VT declined to 1.9 (95%CI, 0.3-11.5). Finally, after adjustment for total C4BP levels, the OR did not change significantly (OR 3.9, 95%CI, 0.7-23.1).

This is in concordance with the finding of a Spearman's Rho of 0.02, indicating there is no significant correlation between free protein S levels and total C4BP.

Free protein S levels below 41 IU/dL in the acute setting were associated with a 3.4-fold increased risk of DVT. This is in agreement with our previous study (8), confirming that a cut-off level of 41 IU/dL is optimal for determining an increased risk of VT. Our results also suggest that this risk is mediated in part by the acute phase response, as the risk of VT decreased after adjustment for CRP (OR 3.6 to 1.9). This risk did not decrease towards 1.0, suggesting that low free protein S levels may also be partially genetically determined (9). Self-reported preceding inflammatory symptoms do not appear to play a role in the mechanism of increasing the risk of VT by lowering levels of free protein S, probably because most of these symptoms were already gone when patients presented at the emergency department. In contrast to the hypothesis that free protein S levels can be modified by C4BP, we did not observe any influence of total C4BP levels on the relation between free protein S and VT, which is in accordance with a previous study (12).

Our results should be interpreted with caution because the risk we found was not statistically significant, probably because our sample was relatively small and free protein S deficiency is a rare phenomenon. As our study was performed in a university hospital setting, our results cannot directly be

translated to the general population. We choose to use referred patients without VT as a control group to reduce the risk of referral and diagnostic bias, and to effectively blind the interviewer for the case-control status of patients. One disadvantage may be that controls were over-similar compared to cases but if this has influenced our results, it would have biased risk estimates towards the null (13). Further, we did only measure free protein S levels once. However, because all total protein S levels were above 65 IU/dL, we did not repeat any measurements, nor performed PROS1 mutation analysis in these patients.

In conclusion, VT was non-significantly associated with low free protein S concentration in patients with clinical symptoms of venous thrombosis, in particular free protein S levels below 41 IU/dL. A part of this association was influenced by the acute phase reaction. Milder degrees of low free protein S were not associated with a risk of VT.

REFERENCES

1. Comp PC, Esmon CT. Recurrent venous thromboembolism in patients with a partial deficiency of protein S. N Engl J Med. 1984;311:1525-8.
2. Seligsohn U, Lubetsky A. Genetic susceptibility to venous thrombosis. N Engl J Med. 2001;344:1222-31.
3. Said JM, Ignjatovic V, Monagle PT, Walker SP, Higgins JR, Brennecke SP. Altered reference ranges for protein C and protein S during early pregnancy: implications for the diagnosis of protein C and protein S deficiency during pregnancy. Thromb Haemost. 2010;103:984-8.
4. Vigano-D'Angelo S, D'Angelo A, Kaufman CE, Jr., Sholer C, Esmon CT, Comp PC. Protein S deficiency occurs in the nephrotic syndrome. Ann Intern Med. 1987;107:42-7.
5. Brouwer JL, Bijl M, Veeger NJ, Kluin-Nelemans HC, van der Meer J. The contribution of inherited and acquired thrombophilic defects, alone or combined with antiphospholipid antibodies, to venous and arterial thromboembolism in patients with systemic lupus erythematosus. Blood. 2004;104:143-8.
6. Lijfering WM, Sprenger HG, Georg RR, van der Meulen PA, van der Meer J. Relationship between progression to AIDS and thrombophilic abnormalities in HIV infection. Clin Chem. 2008;54:1226-33.
7. Mulder R, Tichelaar YI, Sprenger HG, Mulder AB, Lijfering WM. Relationship between cytomegalovirus infection and procoagulant changes in human immunodeficiency virus-infected patients. Clin Microbiol Infect. 2011;17:747-9.
8. Lijfering WM, Mulder R, ten Kate MK, Veeger NJ, Mulder AB, van der Meer J. Clinical relevance of decreased free protein S levels. Results from a retrospective family cohort study involving 1143 relatives. Blood. 2009;113:1225-30.
9. Kearon C, Kahn SR, Agnelli G, Goldhaber S, Raskob GE, Comerota AJ;

American College of Chest Physicians. Antithrombotic therapy for venous thromboembolic disease: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest. 2008;133:454S-545S.

10. Tichelaar YI, Knol HM, Mulder AB, Kluin-Nelemans JC, Lijfering WM. Association between deep vein thrombosis and transient inflammatory signs and symptoms: a case-control study. J Thromb Haemost. 2010;8:1874-6.
11. Comp PC, Doray D, Patton D, Esmon CT. An abnormal plasma distribution of protein S occurs in functional protein S deficiency. Blood. 1986;67:504-8.
12. García de Frutos P, Alim RI, Härdig Y, Zöller B, Dahlbäck B. Differential regulation of alpha and beta chains of C4b-binding protein during acute-phase response resulting in stable plasma levels of free anticoagulant protein S. Blood. 1994;84:815-22.
13. Vandenbroucke JP, Cannegieter SC, Rosendaal FR. Travel and VT: an exercise in thinking about bias. Ann Intern Med. 2009;3:212-3.

Chapter 4

Associations between high factor VIII and low free protein S levels with traditional arterial thrombotic risk factors and their risk on arterial thrombosis: Results from a retrospective family cohort study

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SUMMARY

Whether high factor (F)VIII and low free protein S levels are risk factors for arterial thrombosis is unclarified.

In a post-hoc analysis of a single-centre retrospective family cohort, we determined if these two proteins could increase the risk of arterial thrombosis. In total, 1399 relatives were analysed. Annual incidence in relatives with high FVIII levels was 0.29% (95%CI, 0.22-0.38) compared to 0.13% (95%CI, 0.09-0.19) in relatives with normal FVIII levels. In relatives with low free protein S levels, this risk was 0.26% (95%CI, 0.16-0.40), compared to 0.14% (95%CI, 0.10-0.20) in relatives with normal free protein S levels. Mean FVIII levels adjusted for age and sex were 11 IU/dL, 18 IU/dL, and 21 IU/dL higher in relatives with hypertension, diabetes mellitus, and obesity as compared to relatives without these arterial thrombotic risk factors. Moreover, a dose response relation between increasing FVIII and body mass index was found. None of these associations were shown for free protein S.

High FVIII and low free protein S levels seemed to be mild risk factors for arterial thrombosis. High FVIII levels were particularly observed in relatives with traditional arterial thrombotic risk factors. Free protein S levels were not influenced by these thrombotic risk factors. This assumes that low free protein S levels were genetically determined.

INTRODUCTION

Arterial thrombosis in subjects with arterial thrombotic risk factors is probably mediated by the presence of a prothrombotic and/or inflammatory state (1-5). Factor VIII and free protein S levels are both part of the clotting cascade, but have been reported to be associated with an inflammatory state when levels are high (for factor VIII) (6), or low (for free protein S) (7). Both high factor VIII and low free protein S levels, however, are also partially genetically determined (8, 9). A high level of factor VIII is a well known risk factor for venous thrombosis (10, 11), and possibly for arterial thrombosis as well (11, 12). Whether low free protein S levels are a risk factor for arterial thrombosis is uncertain (13). Most information on low free protein S levels to the risk of arterial thrombosis comes from case reports or small case series (14-16).

We hypothesize that both high factor VIII levels and low free protein S levels increase the risk of arterial thrombosis either through a genetic or acquired link. Presence of a genetic association is assumed when protein levels are not influenced by (acquired) traditional arterial thrombotic risk factors. To test this hypothesis, we performed a retrospective study in a large serie of families with thrombophilic defects to assess the risk of arterial thrombosis for different high factor VIII levels and low free protein S.

MATERIAL AND METHODS

Data retrieval

This is a post-hoc analysis of pooled data from individual subjects of four large retrospective family cohort studies with various thrombophilic index defects from which outcomes have recently been published (17, 18). These studies were performed by three university medical centres (Groningen, Amsterdam and Maastricht). As no central lab was involved, we choose to only include the data obtained from subjects in our centre

(Groningen) to exclude interlaboratory variability in the present study. All studies were performed at the same time and laboratory tests were not changed over time. The first study comprised first-degree relatives (i.e., offspring, siblings, and/or parents) of consecutive patients (probands) with documented venous thrombosis and established hereditary deficiencies of either antithrombin, protein C, or protein S. As the number of antithrombin deficient probands was small, second-degree relatives (i.e., grandparents and/or blood related uncles or aunts) with a deficient parent were also identified. They were enrolled between April 1999 and July 2004. The other three studies comprised first-degree relatives of consecutive patients with venous thrombosis or premature atherosclerosis (< 50 years of age) and the presence of either the prothrombin G20210A mutation, high levels of factor VIII at repeated measurements, or hyperhomocysteinemia. Enrolment started in May 1998 and was completed in July 2004. Approval was obtained from the institutional review board of University Medical Centre Groningen.

Subjects

All relatives, identified by pedigree analysis, were 15 years of age or older and were contacted through the probands. Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. Physicians at our thrombosis outpatient clinic collected detailed information on previous episodes of arterial thrombosis, risk factors for atherosclerosis, and anticoagulant treatment by using a standardised questionnaire (similar for all 4 studies) and reviewing medical records. Clinical data were collected before laboratory testing. Relatives were tested for deficiencies of antithrombin, protein C and protein S, factor V Leiden, prothrombin G20210A, and high levels of factor VIII. In addition, levels of free protein S were measured in most, but not all, relatives due to shortage of stored plasma. Factor VIII levels were measured by one-stage clotting assays (Behring, Marburg, Germany) and were considered high at levels above 150 IU/dL to enable a comparison of results between this study and previous studies of ours (11). Free protein S antigen levels were measured after precipitation of protein S complexed with C4-binding protein with

polyethylene glycol (19). Normal ranges protein S were determined in 393 healthy blood donors, who had no (family) history of venous or arterial thrombosis and were neither pregnant, nor had used oral contraceptives for at least 3 months (20). A free protein S level below 65 IU/dL was considered as low, corresponding with the lower limit of the normal range in healthy volunteers. The CV of the free protein S assay was < 5%. Other laboratory tests, definitions of abnormal results, and criteria for inheritance of natural anticoagulant deficiencies have been described in detail elsewhere (17, 18). To avoid bias, all probands were excluded from the analyses. In addition, relatives with protein S deficiency type I were excluded from analysis when analyzing effects of free protein S. This was done as protein S deficiency is classified into protein S deficiency type I (recognised by decreased levels of both total and free protein S antigen levels) and type III (recognised by decreased free protein S and normal total protein S antigen levels) (21). Protein S deficiency type II, a functional protein S deficiency with reduced APC cofactor activity but normal total and free protein S antigen levels, could not be determined due to the absence of a functional protein S assay in our hospital. Results of hereditary protein S type I deficiency on the risk of arterial thrombosis in this cohort have already been published and were therefore not primarily studied in the present study (22). For similar reasons, we did not primarily study the effect of hereditary antithrombin or protein C deficiency, and prothrombin G20210A on the risk of arterial thrombosis (22, 23). while the number of factor V Leiden carriers was too small in this cohort to provide accurate relative risk estimates. Other studies and meta-analyses already showed that the increase in risk for arterial thrombosis in factor V Leiden carriers is negligible (24). Although previous study questions of ours included whether low free protein S and high factor VIIII levels influence the risk of arterial thrombosis in thrombophilic families (11, 13), these studies did not provide answers to our current hypothesis, that is whether high factor VIII or low free protein S levels are risk factors for arterial thrombosis through an acquired or genetic link.

Definitions

Coronary and peripheral arterial disease had to be symptomatic and angiographically proven, whereas myocardial infarction was diagnosed according to clinical, enzymatic and electrocardiographic criteria. Ischemic stroke was defined as the onset of rapidly developing symptoms and signs of loss of cerebral function which lasted at least 24h and had an apparent vascular cause, as demonstrated by CT or magnetic resonance imaging. If a cerebral event completely resolved within 24h without cerebral lesions at scanning, it was classified as transient ischemic attack (TIA). Known risk factors for arterial thrombosis were recorded and included: hypertension, hyperlipidemia, the presence of diabetes mellitus, smoking habits or obesity defined as body mass index (BMI) > 30 kg/m2.

Statistical analysis

We analysed the absolute risk of first arterial thrombosis in relatives, comparing those who did or did not have high factor VIII levels or low free protein S levels, respectively. We performed a sensitivity analysis where myocardial infarction and ischemic stroke were analyzed separately.

As both factor VIII and free protein S levels are continuous variables, a dichotomous breakdown in the analysis may seem artificial. Therefore, we also analysed factor VIII and free protein S as continuous variables. A further stratification into quartiles to see if there was a dose-response effect, was not feasible due to small numbers. Observation time was defined as the period from the age of 15 years until the first arterial thrombotic episode or until the end of study. The 95% confidence intervals (95% CIs) around the incidence rates were calculated under the Poisson distribution assumption. Relative risks were adjusted for age and sex with Cox regression. As our study cohort consisted of subjects from thrombophilic families and were therefore prone to have multiple thrombophilic defects (24, 25), we adjusted for antithrombin, protein C or protein S type I deficiency, factor V Leiden and prothrombin G20210A with stepwise Cox regression to provide as homogenous risk estimates as possible. To account for the non-randomness

of the relatives analysed, relative risks were also adjusted for clustering of events within families by using random-effects Cox regression and the robust sandwich method in Stata.

Linear regression was used to determine the relation between factor VIII levels and free protein S levels, respectively, combined with traditional arterial thrombotic risk factors. Adjustments were made for age and sex.

A cumulative distribution curve is a graphical representation in which two continuous variables can directly be compared. They were constructed to visualize a possible relationship between factor VIII and BMI, and free protein S and BMI and the occurrence of arterial thrombosis.

Continuous variables were expressed as mean values and standard deviations; categorical data as counts and percentages. Differences between groups were evaluated by the Student *t* test and by Fisher exact test for categorical variables. A 2-tailed *P*-value of less than .05 indicated statistical significance. The statistical software used was SPSS version 16.0 (SPSS, Chicago, Illinois, United States) and Stata version 10.1 (Stata Corp., College Station, Texas).

RESULTS

Our study cohort comprised 2451 relatives aged 15 years or older, of 380 probands (Figure 1). Of relatives, 518 (21%) had died before the start of the study. Another 465 relatives did not participate because of various reasons, including refusal, inability to give informed consent, or residence outside The Netherlands (exclusion rate 24%). Of 1468 relatives tested for thrombophilia, 1399 were analysed on factor VIII (missing laboratory data, n=69) and 1143 were analysed on free protein S (94 relatives excluded with protein S type I deficiency and 231 relatives with missing laboratory data). Forty-six percent were male (Table 1). Mean age at enrolment was 45 years.

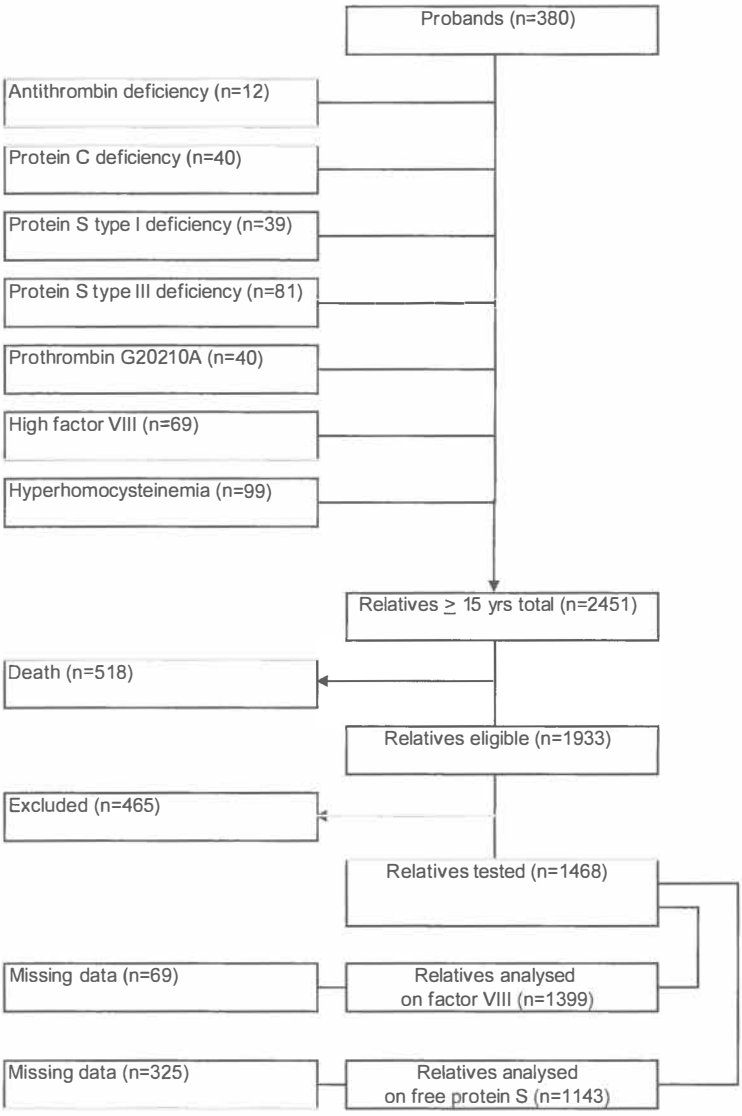

Figure 1. Flow diagram of the family cohort.

Mean observation period was 30 years. Arterial thrombotic events were documented in 86 relatives (6%) at a mean age of 57 years. In relatives with high factor VIII levels mean age of occurrence of arterial thrombosis was 60 years. Mean age of arterial thrombosis in relatives with low free protein S levels was 55 years. Mean factor VIII level was 146 IU/dL and mean free protein S level was 80 IU/dL.

Table 1 Characteristics of 1399 relatives of probands with a thrombophilic defect

Male, n (%)	648 (46)
Age at enrolment, mean (SD)	45 (17)
Arterial thrombosis, n (%)	86 (6)
Age at onset arterial thrombosis, mean (SD)	57 (13)
Classification	
Myocardial infarction, n (%)	32 (37)
Ischemic stroke, n (%)	21 (24)
Transient ischemic attack, n (%)	17 (20)
Peripheral arterial thrombotic event, n (%)	16 (19)
Long term vitamin K antagonists, n (%)	38 (3)
Oral contraceptive use (% women), n (%)	210 (28)
Thrombophilia	
Factor VIII, mean (SD)	146 (53)
Factor VIII > 150 IU/dL, n (%)	547 (39)
Free protein S*, mean (SD)	80 (20)
Free protein S < 65 IU/dL*, n (%)	259 (23)
Arterial thrombotic risk factors	
Hypertension, n (%)	236 (17)
Hyperlipidemia, n (%)	162 (12)
Diabetes mellitus, n (%)	58 (4)
Previous smokers, n (%)	295 (21)
Obesity (body mass index ≥ 30 kg/m^2), n (%)	185 (13)

* 94 relatives with protein S type I deficiency excluded, total tested relatives 1143.

Annual incidence of arterial thrombosis in relatives with high factor VIII levels was 0.29% (95% CI, 0.22-0.38) compared to 0.13% (95% CI, 0.09-0.19) in relatives with normal factor VIII levels, crude relative risk 2.2 (95% CI, 1.4-3.4) (Table 2). When end-point myocardial infarction was chosen, this crude relative risk was 3.6 (95% CI, 1.6-8.0). For ischemic stroke, this crude relative risk was 2.0 (95% CI, 0.8-4.8). Adjusted for age, sex and clustering of events within families, relative risk of arterial thrombosis in relatives

Table 2 Risk of arterial thrombosis in relatives with high factor VIII levels or low free protein S levels

	Observation years (relatives)	Relatives with event	Annual incidence, % (95% CI)	Crude relative risk (95% CI)	Adjusted relative risk (95% CI)*	Adjusted relative risk (95% CI)†	Adjusted relative risk (95% CI)‡	Adjusted relative risk (95% CI), adjusted for age, sex and				
								Antithrombin deficiency	Protein C deficiency	Protein S type I deficiency	Factor V Leiden	Prothrombin G20210A
Factor VIII	22465	30	0.13	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
< 150 IU/dL	(n=852)		(0.09-0.19)									
Factor VIII	19388	56	0.29	2.2	1.5	1.6	1.4	1.5	1.7	1.8	1.7	1.5
> 150 IU/dL	(n=547)		(0.22-0.38)	(1.4-3.4)	(0.9-2.8)	(0.9-2.8)	(0.8-2.3)	(0.9-2.4)	(1.0-3.0)	(1.0-3.0)	(1.0-3.0)	(0.9-2.4)
Free protein S	26405	38	0.14	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
> 65 IU/dL	(n=884)		(0.10-0.20)									
Free protein S	7968	21	0.26	1.8	1.7	1.8	1.7	1.7	1.7	NA	1.7	1.7
< 65 IU/dL	(n=259)		(0.16-0.40)	(1.1-3.1)	(1.1-3.1)	(1.0-3.2)	(1.0-2.9)	(1.0-3.0)	(1.0-3.0)		(1.0-3.0)	(1.0-3.0)

* Relative risk adjusted for age, sex and clustering of events within families.

† Exluding estrogen users (n=210), adjusted for age and sex and clustering of events within families.

‡ Relative risk adjusted for age, sex and traditional arterial thrombotic risk factors.

NA denotes not applicable; protein S type I deficient relatives were excluded when analysing risk of arterial thrombosis for decreased free protein S levels.

with high factor VIII levels as compared to relatives with normal factor VIII levels was 1.5 (95% CI, 0.9-2.5). Age was the main determinant in the model that influenced this adjusted risk. After traditional arterial thrombotic risk factors were added in this model, relative risk was 1.4 (95% CI, 0.8-2.3).

In relatives with low free protein S levels, annual incidence of arterial thrombosis was 0.26% (95% CI, 0.16-0.40), compared to 0.14% (95% CI, 0.10-0.20) in relatives with normal free protein S levels, crude relative risk 1.8 (95% CI, 1.1-3.1). When end-point myocardial infarction was chosen, this crude relative risk was 1.7 (95% CI, 0.7-4.2). For ischemic stroke, this crude relative risk was 2.1 (95% CI, 0.8-5.7). Adjusted for age, sex and clustering of events within families, relative risk of arterial thrombosis in relatives with low free protein S levels as compared to relatives with normal free protein S levels was 1.7 (95% CI, 1.1-3.1). After traditional arterial thrombotic risk factors were added in this model, relative risk was 1.7 (95% CI, 1.0-2.9). As oral contraceptives may increase factor VIII levels and decrease free protein S levels (26), we performed a sensitivity analysis excluding all women who used oral contraceptives at time of enrolment. This did not change outcomes. Adjustments for various thrombophilic defects by stepwise Cox regression did also not change outcomes. When factor VIII and free protein S were analysed as a continuous variable in a Cox proportional-hazards model, the age- sex and clustering of events within families adjusted relative risk of arterial thrombosis was 1.003 (95% CI, 0.999-1.007) for each increase of 1 IU/dL in the level of factor VIII and 0.989 (95% CI, 0.978-0.998) for each increase of 1 IU/dL in the level of free protein S.

Cumulative distribution functions of free protein S levels and increasing factor VIII levels were overlying (Figure 2). Hence, no relationship between decreasing free protein S levels and increasing factor VIII levels was observed.

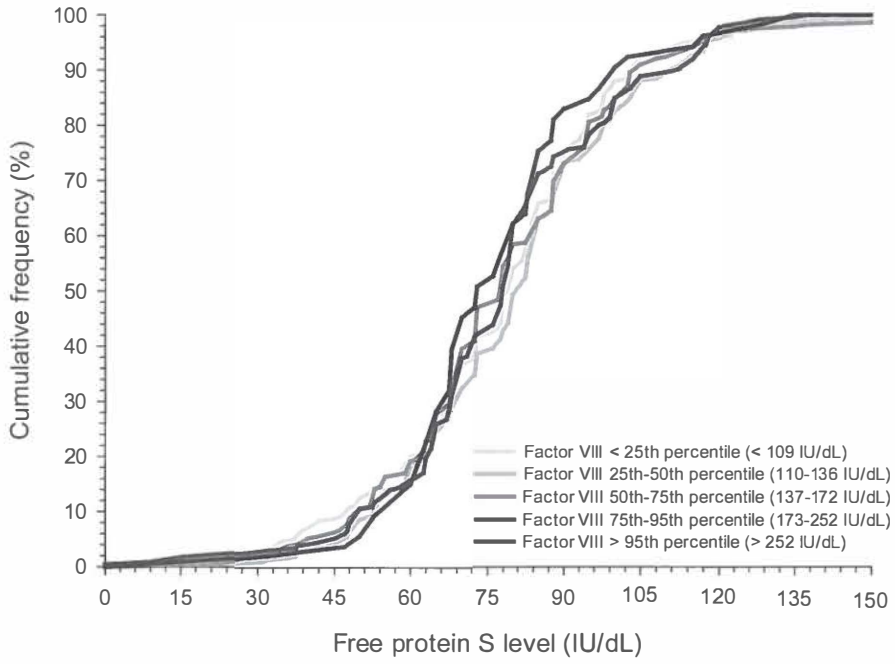

Figure 2. Cumulative distribution function of free protein S levels in relatives of probands with a thrombophilic defect.

Table 3 shows mean factor VIII levels in relatives with hypertension, hyperlipidemia, diabetes mellitus, previous smokers, or who were obese. Relatives with one of these traditional arterial thrombotic risk factors had mean factor VIII levels that were 24 IU/dL, 17 IU/dL, 31 IU/dL, 10 IU/dL and 26 IU/dL higher compared to relatives without exposure to these risk factors. After adjustment of age and sex, these levels were 11 IU/dL, 5 IU/dL, 18 IU/dL, 1 IU/dL and 21 IU/dL higher and still statistically significant for hypertension, diabetes mellitus, and obesity. Mean free protein S levels were similar in relatives who either were or were not exposed to traditional arterial thrombotic risk factors, possibly excepted for relatives with obesity, where obese relatives appeared to have higher free protein S levels than non-obese relatives.

Because we had continous data on BMI, we could make cumulative distribution functions of factor VIII levels and free protein S levels on BMI. As shown in Figure 3A, an increase in factor VIII was associated with higher BMI. As no such relation was observed for free protein S levels (Figure 3B) suggests that the earlier found increase of free protein S levels in obese versus non-obese relatives is a result of chance and not a real association. Relative risk, adjusted for age, sex and clustering of events within families, for arterial thrombosis in relatives with hypertension was 1.8 (95% CI, 1.2-2.9) compared to relatives with normotension. This risk remained unchanged after further adjustment for factor VIII. Relative risk for arterial thrombosis in relatives with hyperlipidemia, diabetes mellitus, previous smokers and obese relatives were 2.8 (95% CI, 1.8-4.4), 1.2 (95% CI, 0.6-2.5), 1.5 (95% CI, 1.0-2.2) and 1.5 (95% CI, 0.8-2.9) adjusted for age, sex and clustering of events within families and compared to relatives without the exposure. Extra adjustment for factor VIII did also not change these outcomes.

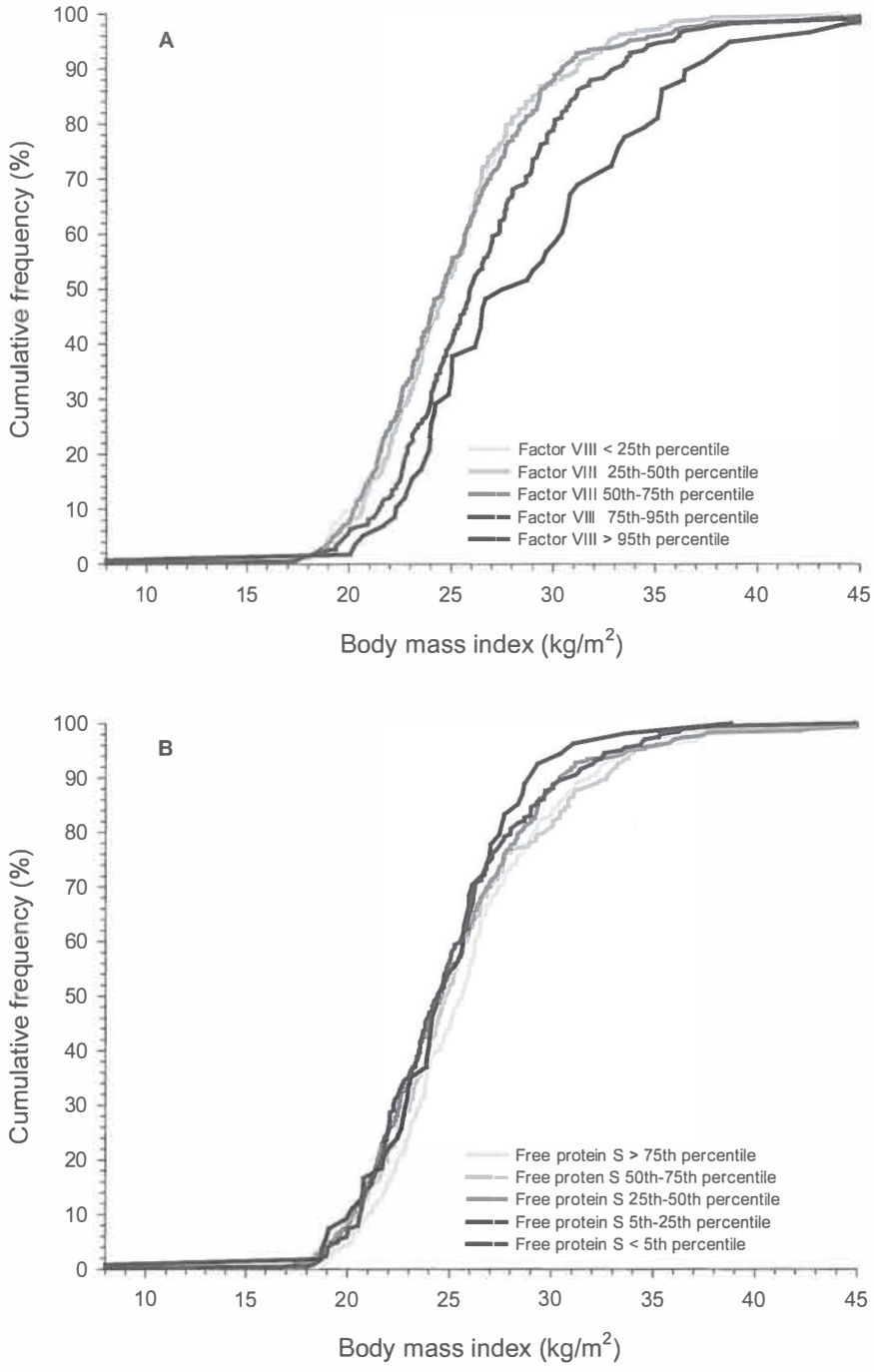

Figure 3. Cumulative distribution function of factor VIII levels (A) and free protein S levels (B) in relatives of probands with a thrombophilic defect according to body mass index.

DISCUSSION

This retrospective family study showed an approximately 2-fold increased risk of arterial thrombosis in relatives with high factor VIII levels or low free protein S levels. Age had a strong effect on this risk in relatives with high factor VIII levels, as after adjustment the risk dropped to 1.5. The risk of arterial thrombosis in relatives with low free protein S remained, however, unchanged after adjustment for age. Furthermore, factor VIII levels were higher in relatives with traditional arterial thrombotic risk factors, whereas no such association was observed for free protein S levels. Somewhat surprisingly, we could not demonstrate that higher factor VIII levels were associated with lower free protein S levels. This correlation could be expected as both thrombophilic abnormalities are associated with chronic inflammation (7, 27), and chronic inflammation is considered to be a risk factor for arterial thrombosis (3, 4, 27). On the other hand, that this association was not shown could be a consequence of the family design of our study. Although genotype-phenotype associations in relatives with low free protein S levels were not determined in this study, it is likely that a genetic factor is involved, as free protein S levels and arterial thrombotic risk were not influenced by age, while no association between free protein S levels with traditional arterial risk factors was shown. In addition, mean free protein S levels in this study (80 IU/dL) showed a left shift compared with the normal population (mean 100 IU/dL), which is likely a result of including thrombophilic families (17). Accordingly, one might expect the presence of low free protein S levels in relatives of patients with arterial thrombosis who have a family history of venous thrombosis or premature atherosclerosis. However, it cannot be concluded from this study if testing for free protein S is useful for primary or secondary prevention of arterial thrombosis.

Several methodologic aspects of our study warrant comment. First, because the study had a retrospective design, where traditional arterial thrombotic

risk factors were self-reported and/or derived from medical records, it is possible that misclassification occurred. This might have led to slightly lower risks and differences conferred by traditional arterial thrombotic risk factors, but was probably reduced by using a standardised questionnaire. Second, height and weight were self-reported. As in general subjects with underweight tend to overreport their body weight, while subjects with overweight tend to underreport their body weight (28), actual risks and differences could be somewhat higher than reported if this phenomenon occurred. Third, referral bias may have been introduced by the university hospital setting. However, this was probably reduced by testing consecutive patients with thrombosis. Fourth, absolute risk estimates for arterial thrombosis were low in our study cohort. This clearly is a result of enrolling young relatives in the study as mean age at enrolment was 45 years in our cohort. Although generalizibility of our results for this reason is hampered (but also because of the family cohort design) a family study of young participants is probably ideal to determine whether genetic variants are involved for arterial thrombotic disease occurrence as increasing age is strongly associated with an increased risk of arterial thrombosis (25). Fifth, although we used a large study cohort and long follow up period, we only observed a relative small number of arterial events (total n=86) that resulted in relatively wide confidence intervals. Hence, our study results should be interpreted with caution.

Finally, in this retrospective study, factor VIII levels were influenced by age, diabetes mellitus, obesity and hypertension. Causal inference of high factor VIII levels on arterial thrombotic risk can therefore not be inferred. Nevertheless, it might be interesting for future studies to determine why factor VIII levels increase with age, hypertension, diabetes mellitus and obesity. Shear stress (for hypertension) or endothelial damage (for increasing age) might explain these findings, but these hypotheses have, as far as we know, not been studied yet in humans. Furthermore, ABO blood group plays a significant role on factor VIII levels. This topic could not be covered in our study, as blood group was not measured.

Although free protein S levels were stable over time, and were not influenced by traditional arterial thrombotic risk factors, which assumes that low free protein S levels are genetically determined, we cannot exclude the possibility that this is based on residual confounding. Whether low free protein S levels are genetically determined can only be inferred with certainty from genotype-phenotype studies. Based on the present finding that low free protein S levels are associated with arterial thrombosis, and based on our similar finding in a previous study, but then on venous thrombotic risk, which was independent of traditional venous thrombotic risk factor (17), could provide rationale for future studies to perform such a genotype-phenotype study.

We did not use a normal range of free protein S that was stratified on sex and age, although it is known that this is lower in premenopasal women than in men (20).

However, adjustment for sex and age did not change our outcomes. Furthermore, oral contraceptive use and hormonal replacement therapy decrease free protein S levels (26) and are known risk factors for venous thrombosis as well (29). Therefore, we performed a sensitivity analysis excluding all women who used oral contraceptives at time of enrollment. This did not change outcomes.

In conclusion, both high factor VIII levels and low free protein S levels seemed to be a mild risk factor for arterial thrombosis in thrombophilic families. High factor VIII levels were particularly observed in relatives with traditional arterial thrombotic risk factors. Hence, it is questionable if a high factor VIII level itself increases risk of arterial thrombosis, or if this risk is explained by other, factor VIII associated arterial thrombotic risk factors, such as increasing age or hypertension. Free protein S levels were not influenced by traditional arterial thrombotic risk factors, which assumes that low free protein S levels were genetically determined. Larger studies on this issue are required.

REFERENCES

1. Ruggeri ZM. Platelets in atherothrombosis. Nat Med. 2002;8:1227-34.
2. Vanhoutte PM. Endothelial dysfunction: the first step toward coronary arteriosclerosis. Circ J. 2009;73:595-601.
3. Gawaz M, Langer H, May AE. Platelets in inflammation and atherogenesis. J Clin Invest. 2005;115:3378-84.
4. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999;340:115-26.
5. Tracy RP. Inflammation in cardiovascular disease: cart, horse, or both? Circulation. 1998;97:2000-2.
6. Rossouw JE, Cushman M, Greenland P, Lloyd-Jones DM, Bray P, Kooperberg C, Pettinger M, Robinson J, Hendrix S, Hsia J. Inflammatory, lipid, thrombotic, and genetic markers of coronary heart disease risk in the women's health initiative trials of hormone therapy. Arch Intern Med. 2008;168:2245-53.
7. Anderson HA, Maylock CA, Williams JA, Paweletz CP, Shu HJ, Shacter E. Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. Nature Immunology. 2003;4:87-91.
8. Kamphuisen PW, Houwing-Duistermaat JJ, van Houwelingen HC, Eikenboom JC, Bertina RM, Rosendaal FR. Familial clustering of factor VIII and von Willebrand factor levels. Thromb Haemost. 1998;79:323-7.
9. Ten Kate MK, Platteel M, Mulder R, Terpstra P, Nicolaes GA, Reitsma PH, van der Steege G, van der Meer J. PROS1 analysis in 87 pedigrees with hereditary protein S deficiency demonstrates striking genotype-phenotype associations. Hum Mutat. 2008;29:939-47.
10. Koster T, Blann AD, Briet E, Vandenbroucke JP, Rosendaal FR. Role of clotting factor VIII in effect of von Willebrand factor on occurrence of deep-vein thrombosis. Lancet. 1995;345:152-5.

11. Bank I, Libourel EJ, Middeldorp S, Hamulyák K, van Pampus EC, Koopman MM, Prins MH, van der Meer J, Büller HR. Elevated levels of FVIII:C within families are associated with an increased risk for venous and arterial thrombosis. J Thromb Haemost. 2005;3:79-84.

12. Folsom AR, Wu KK, Rosamond WD, Sharrett AR, Chambless LE. Prospective study of hemostatic factors and incidence of coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) Study. Circulation. 1997;96:1102-8.

13. Brouwer JL, Veeger NJ, van der Schaaf W, Kluin-Nelemans HC, van der Meer J. Difference in absolute risk of venous and arterial thrombosis between familial protein S deficiency type I and type III. Results from a family cohort study to assess the clinical impact of a laboratory test-based classification. Br J Haematol. 2005;128:703-10.

14. Beattie S, Norton M, Doll D. Coronary thrombosis associated with inherited protein S deficiency: a case report. Heart Lung. 1997;26:76-9.

15. Horowitz IN, Galvis AG, Gomperts ED. Arterial thrombosis and protein S deficiency. J Pediatr. 1992;121:934-7.

16. Zimmerman AA, Watson RS, Williams JK. Protein S deficiency presenting as an acute postoperative arterial thrombosis in a four-year-old child. Anesth Analg. 1999;88:535-7.

17. Lijfering WM, Mulder R, ten Kate MK, Veeger NJ, Mulder AB, van der Meer J. Clinical relevance of decreased free protein S levels. Results from a retrospective family cohort study involving 1143 relatives. Blood. 2009;113:1225-30.

18. Lijfering WM, Brouwer JL, Veeger NJ, Bank I, Coppens M, Middeldorp S, Hamulyák K, Prins MH, Büller HR, van der Meer J. Selective testing for thrombophilia in patients with first venous thrombosis: results from a retrospective family cohort study on absolute thrombotic risk

for currently known thrombophilic defects in 2479 relatives. Blood. 2009;113:5314-22.

19. Comp PC, Thurnau GR, Welsh J, Esmon CT. Functional and immunologic protein S levels are decreased during pregnancy. Blood. 1986;68:881-5.

20. Henkens CM, Bom VJ, van der Schaaf W, Pelsma PM, Sibinga CT, de Kam PJ, van der Meer J. Plasma levels of protein S, protein C, and factor X: effects of sex, hormonal state and age. Thromb Haemost. 1995;74:1271-5.

21. Dahlbäck B. The tale of protein S and C4b-binding protein, a story of affection. Thromb Haemost. 2007;98:90-6.

22. Mahmoodi BK, Brouwer JL, Veeger NJ, van der Meer J. Hereditary deficiency of protein C or protein S confers increased risk of arterial thromboembolic events at a young age: results from a large family cohort study. Circulation. 2008;118:1659-67.

23. Bank I, Libourel EJ, Middeldorp S, Van Pampus EC, Koopman MM, Hamulyák K, Prins MH, van der Meer J, Büller HR. Prothrombin 20210A mutation: a mild risk factor for venous thromboembolism but not for arterial thrombotic disease and pregnancy-related complications in a family study. Arch Intern Med. 2004;164:1932-7.

24. Juul K, Tybjaerg-Hansen A, Steffensen R, Kofoed S, Jensen G, Nordestgaard BG. Factor V Leiden: The Copenhagen City Heart Study and 2 meta-analyses. Blood. 2002;100:3-10.

25. Rosamond W, Flegal K, Furie K, Go A, Greenlund K, Haase N, Hailpern SM, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell C, Roger V, Sorlie P, Steinberger J, Thom T, Wilson M, Hong Y; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation. 2008;117:e25-146.

26. Bloemenkamp KW, Rosendaal FR, Helmerhorst FM, Koster T, Bertina RM, Vandenbroucke JP. Hemostatic effects of oral contraceptives in women who developed deep-vein thrombosis while using oral contraceptives. Thromb Haemost. 1998;80:382-7.

27. Bhagat K, Vallance P. Inflammatory cytokines impair endothelium-dependent dilatation in human veins in vivo. Circulation. 1997;96:3042-7.

28. Gunnell D, Berney L, Holland P, Maynard M, Blane D, Frankel S, Smith GD. How accurately are height, weight and leg length reported by the elderly, and how closely are they related to measurements recorded in childhood? Int J Epidemiol. 2000;29:456-64.

29. Vandenbroucke JP, Rosing J, Bloemenkamp KW, Middeldorp S, Helmerhorst FM, Bouma BN, Rosendaal FR. Oral contraceptives and the risk of venous thrombosis. N Engl J Med. 2001;344:1527-35.

Chapter 5

Low cut-off values increase diagnostic performance of protein S assays

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SUMMARY

Conflicting data have been reported on the accuracy of protein S assays for detection of hereditary protein S deficiency. In this study we assessed the diagnostic performance of two total protein S antigen assays, four free protein S assays and three protein S activity assays in a group of 28 heterozygous carriers of mutations in PROS1 and 165 control subjects. Several control groups were formed, one of healthy volunteers and because protein S levels are influenced by oral contraception and pregnancy, and assays measuring protein S activity may be influenced by the presence of the factor V Leiden mutation, we also investigated the influences of these factors.

All nine protein S assays detected significantly reduced protein S levels in subjects with a PROS1 mutation. Eight out of nine protein S assays showed a 100% sensitivity and 100% specificity to detect heterozygous carriers of mutations in PROS1 with values far below the lower limit of the reference values obtained from healthy volunteers. Low specificities were found in subjects with a factor V Leiden mutation and in pregnant women. At lower cut-off levels, equal to the highest protein S value found in heterozygous carriers of mutations in PROS1, the specificity considerably increased in these subjects.

When using low cut-off levels equal to the highest protein S value found in heterozygous carriers of mutations in PROS1, ensuring 100% sensitivity, the specificity in all study groups increases considerably, by which misclassification can be maximally avoided.

INTRODUCTION

Protein S is a vitamin K-dependent glycoprotein that functions as a non-enzymatic cofactor for activated protein C (APC) in the proteolytic degradation of activated coagulation factors V and VIII (1-3). Protein S circulates in plasma in both the bound and free form. Approximately 60% of the total amount of protein S forms a 1:1 stoichiometry with the ß-chain of complement component C4-binding protein (C4BP) (4).

Based on the plasma concentration of total protein S, free protein S, and APC cofactor activity, protein S deficiency is categorized into three subtypes. Protein S deficiency type I is characterized by decreased levels of both total and free protein S. Type II is a functional deficiency with normal protein S antigen levels, but reduced APC cofactor activity. Protein S deficiency type III is characterized by normal levels of total protein S, but decreased levels of free protein S.

Whereas protein S deficiency is a relatively rare autosomal dominant disorder with an estimated prevalence of 0.03-0.13% in the general population, it is found in 2-4% of patients with venous thrombosis (5, 6).

Type I protein S deficiency is an established risk factor for venous thromboembolism with an annual incidence of venous thrombosis ranging from 1.0 to 3.1% (7-10). Limited and conflicting data have been reported on the risk of thrombosis associated with type II and III deficiencies (11-15). In recent studies, we demonstrated that decreased free protein S levels are a risk factor for venous thrombosis in subjects only with levels far below the lower limit of the reference range obtained from healthy volunteers (16, 17). Plasma protein S can be quantified by many different immunological and functional protein S assays, all with their own reference range and sensitivity and specificity profile. Furthermore, due to variations in protein S levels related to age, gender, and acquired conditions, subjects can be misdiagnosed (18-23).

In this study, we evaluated the diagnostic performance of nine different protein S assays in a well-defined reference group of protein S-deficient subjects with a genetically-confirmed heterozygous PROS1 mutation and belonging to thrombophilic families. Controls consisted of healthy volunteers and because protein S levels are influenced by the use of oral contraception and pregnancy (18-23) and assays measuring protein S activity may be influenced by the presence of factor V Leiden mutation (24, 25), we also investigated the potential influences of these accompanying conditions. To increase the specificity, we also selected the highest protein S value found with the different assays in the heterozygous carriers of mutations in PROS1, as cut-off value, still ensuring a 100% sensitivity.

MATERIALS AND METHODS

Subjects

The study group consisted of 28 subjects, belonging to families of probands who had experienced venous thromboembolism, and having a causative PROS1 mutation (PSD), confirmed by direct sequencing in a previous study (26). Subjects with the protein S Heerlen allele were excluded. The control groups consisted of 35 heterozygous factor V Leiden carriers (FVLhet) and 18 homozygous factor V Leiden carriers (FVLhom). These subjects were selected from previous family cohort studies. Individuals on anticoagulation therapy were not included. In addition, healthy women, who used second generation oral contraceptives for at least 3 months (OC, n=30), healthy women in the third trimester of their pregnancy (Pregnant, n=35) and healthy volunteers (HV, 24 men and 23 women) were included as controls. Age and sex of each study group are documented in Table 1. In none of the controls a concomitant PROS1 mutation was present.

Table 1. Age and sex characteristics of the study population

	HV	PSD	FVLhet	FVLhom	OC	Pregnant
N	47	28	35	18	30	35
Male, %	51	46	54	28	NA	NA
Age, y	42 (11)	37 (17)	44 (17)	41 (12)	25 (5)	32 (5)

PSD indicates heterozygous carriers of mutations in PROS1. Control groups consisting of healthy volunteers (HV), subjects with the factor V Leiden mutation (FVL) heterozygous (het) or homozygous (hom), women using oral contraception (OC) and pregnant women (pregnant). All results shown correspond to the mean. The SD is given in brackets. NA indicates not applicable.

Blood collection

Venous blood samples were anticoagulated with 1:10 volume of 0.109 mol/l trisodium citrate. Platelet-poor plasma was prepared by centrifugation at 2500 x g for 15 minutes, aliquoted and immediately frozen at -80°C and analysed later after rapidly thawing at 37 °C.

Protein S assays

Total protein S antigen levels were measured with two different enzyme-linked immunosorbent assays (ELISA), i.e. with reagents obtained from DAKO, Glostrup, Denmark (TPS) and with reagents (Reaads® Protein S Antigen) obtained from Corgenix, Broomfield, USA (PA).

Free protein S antigen levels were measured with four different assays; i.e. an ELISA with reagents obtained from DAKO, Glostrup, Denmark after precipitation of protein S bound to C4BP with 3.75% PEG 6000 (FPS), an ELISA with reagents (Reaads® Monoclonal Free Protein S Antigen) obtained from Corgenix, Broomfield, USA (MFPA), and two assays based on latex beads containing C4BP with reagents (HemosIL™) obtained from Instrumentation Laboratory, Milano, Italy (HemosIL) and reagents (STA® - Liatest® Free Protein S) obtained from Roche Diagnostics, Almere, the Netherlands (Liatest). The reference for free protein S levels was set at 100%.

Protein S activity was measured with three different assays; i.e. with reagents obtained from Siemens, Marburg, Germany (PSact), reagents (Cryocheck quantitative Protein S Clotting Assay CLOT S™) obtained from Precision Biologic, Dartmouth, Canada (CLOT S), and reagents (STA Protein S Clotting) obtained from Roche Diagnostics, Almere, the Netherlands (STA-CLOT).

Our in-house TPS and FPS assays were standardised using a home-made normal plasma pool, prepared in our laboratory and precisions were determined with the same (un-)diluted normal plasma pool. All other seven protein S assays were standardised using commercial calibrators, included in the kits of the manufacturers and precisions were determined with commercial, lyophilised plasma controls, also included in the kits.

The Liatest and STA-CLOT assays were performed using a STA-R Evolution® (Roche Diagnostics, Almere, the Netherlands). The HemosIL and CLOT S assays were performed using a CA-7000® system (Sysmex Corporation, Siemens, Marburg, Germany). The PSact assay was performed using a BCS® system (Siemens, Marburg, Germany).

All tests were performed by the same technician using one lot of reagents.

Factor V Leiden

Genomic DNA was prepared using a commercial DNA extraction kit (Qiamp, Qiagen SA, Courtaboeuf, France). Genetic analysis of the factor V R506Q mutation was performed by a qPCR (27).

Statistical Analysis

Continuous variables were expressed as mean ± standard deviation (SD) and categorical data as count and percentage. Differences between groups were calculated with the Student's *t*-test. A two-tailed *P*-value of less than 0.05 indicated significance. Analyses were performed using SPSS software, version 16.0.2 (SPSS Inc., Chicago, IL).

RESULTS

Precision

For each protein S assay we evaluated the within-run (CVintra) and between-run (CVinter) precision with a normal and abnormal control sample (Table 2). Control samples were assayed 10 times on a single run to calculate the within-run precision. The between-run precision was based on duplicate runs on 5 consecutive days. The CV was expressed as percentage of the mean value of the protein S levels determined in the different normal and abnormal control samples that were used for the CVintra and CVinter determination.

For all nine protein S assays, the CVintra and CVinter values were below 10% when using the normal control sample. Two assays showed CVinter results higher than 10% with the abnormal control sample, i.e. the MFPA assay (12.4%) and the PA assay (10.9%).

Protein S levels

In total, 193 subjects aged 15 years or older were tested with nine different protein S assays. Boxplots showing the distribution of protein S levels in all study groups are presented in Figure 1.

All nine assays detected significantly ($P<0.05$) reduced protein S levels in the group of heterozygous carriers of mutations in PROS1, as compared with the healthy volunteers.

Healthy women tended to present with lower total protein S, free protein S and protein S activity levels than men, which reached statistically significance in two free protein S assays, i.e. the Liatest and HemosIL assays, and two protein S activity assays, i.e. the STA-CLOT and CLOT S assays (data not shown). No statistically significant gender differences in protein S levels were found for the PROS1 mutation carriers.

Table 2. Analytical validation of 9 protein S assays

	Normal		Abnormal		
Test	CVintra	CVinter	CVintra	CVinter	PS mean (IU/dL)
TPS	1.9	4.9	3.4	2.7	26
PA	4.4	8.2	2.9	10.9	50
FPS	3.1	1.9	4.3	4.4	25
MFPA	5.3	3.2	3.1	12.4	46
Liatest	1.7	3.7	1.8	1.6	26
HemosIL	1.9	5.8	3.3	2.9	26
STA-CLOT	1.3	3.7	2.6	3.9	33
PSact	0.7	4.0	0.9	6.9	29
CLOT S	2.4	6.4	5.5	4.1	49

Within-day (CVintra) and between-day (CVinter) coefficient of variation (CV). The CVs were expressed as percentage of the mean value of the protein S levels determined in the different normal and abnormal (protein S mean) control samples. All data are presented as percentage. Analytical validation of total protein S assay with reagents obtained from DAKO (TPS), total protein S assay from Corgenix (PA), free protein S assay with reagents obtained from DAKO (FPS), free protein S assay from Corgenix (MFPA), free protein S assay from Roche Diagnostics (Liatest), free protein S assay from Instrumentation Laboratory (HemosIL), protein S activity assay from Roche Diagnostics (STA-CLOT), protein S activity assay from Siemens (PSact), protein S activity assay from Precision Biologic (CLOT S).

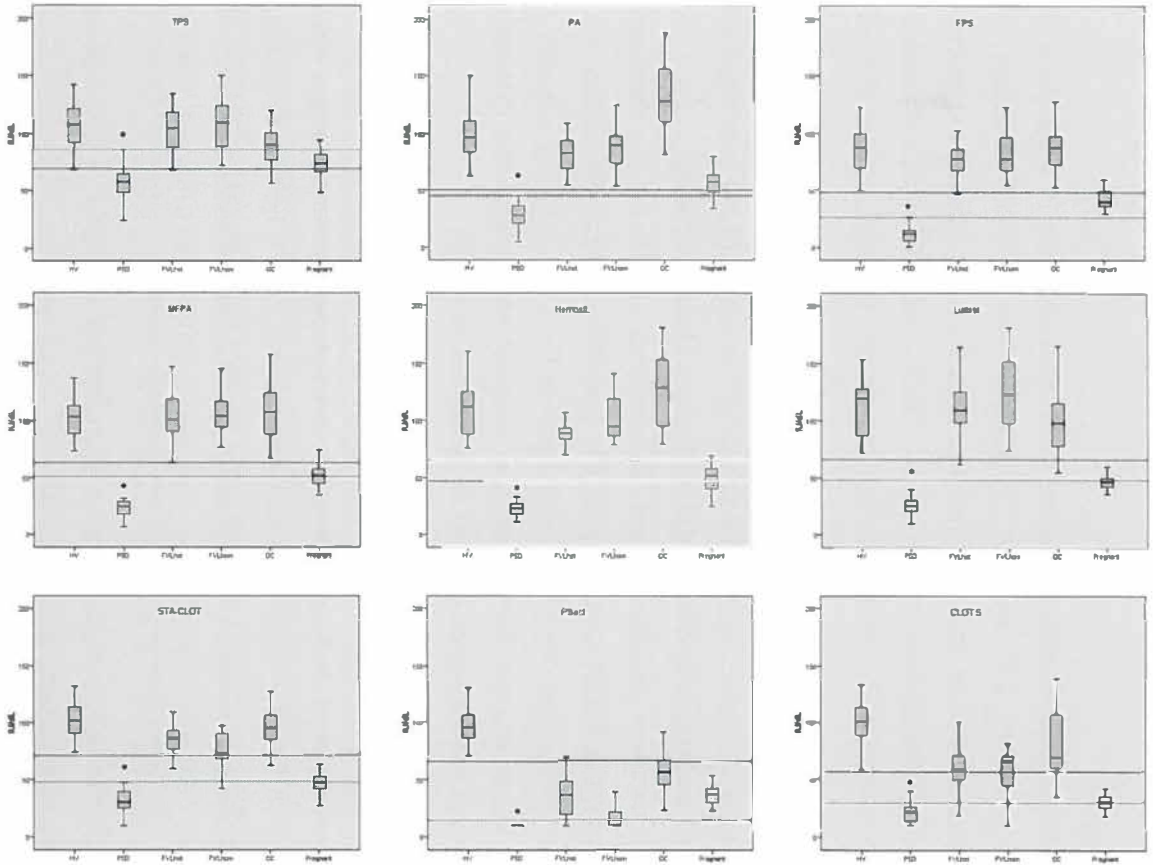

Figure 1. Protein S levels measured with nine different protein S assays.

Protein S levels measured with nine different protein S assays in heterozygous carriers of mutations in PROS1 (PSD) and control groups consisting of healthy volunteers (HV), subjects with the factor V Leiden mutation (FVL) heterozygous (het) and homozygous (hom), women using oral contraception (OC) and pregnant women (pregnant). Bold lines represent the mean - 2SD for healthy volunteers and dashed lines represent levels equal to the highest protein S value found in heterozygous carriers of mutations in PROS1. * Denotes $P<0.05$. Boxplots of total protein S assay with reagents obtained from DAKO (TPS), total protein S assay from Corgenix (PA), free protein S assay with reagents obtained from DAKO (FPS), free protein S assay from Corgenix (MFPA), free protein S assay from Instrumentation Laboratory (HemosIL), free protein S assay from Roche Diagnostics (Liatest), protein S activity assay from Roche Diagnostics (STA-CLOT), protein S activity assay from Siemens (PSact), protein S activity assay from Precision Biologic (CLOT S).

Sensitivity

With eight out of nine assays, all heterozygous carriers of mutations in PROS1 showed protein S values below the lower limit of the reference range obtained with the healthy volunteers (mean - 2SD), corresponding with a sensitivity of 100%. The TPS assay performed less well with a 93% sensitivity, as total protein S levels (i.e. 74 and 86 IU/dL) were above the lower limit of the reference range (71 IU/dL) in two heterozygous carriers of mutations in PROS1.

Specificity

Significantly reduced protein S levels were also obtained in subjects with a heterozygous (PA, FPS, HemosIL, STA-CLOT, PSact, and CLOT S assays) or homozygous FV Leiden mutation (all 3 protein S activity assays), in women using oral contraceptives (TPS, Liatest, PSact, and CLOT S assays) and in pregnant women in the third trimester (all protein S assays, except the TPS assay).

To increase the specificity for these groups, we selected as cut-off value the highest protein S value, found with the different assays in the heterozygous carriers of mutations in PROS1, still ensuring a 100% sensitivity.

Table 3 shows the specificities and false positive results for the diagnosis of heterozygous PROS1 mutation for the two different cut-off values; i.e. the lower limit of the reference range obtained with the healthy volunteers (mean - 2SD); and the highest protein S value, found with the different assays in the heterozygous carriers of mutations in PROS1.

Total protein S assay with reagents obtained from DAKO (TPS)

At a cut-off value of 71 IU/dL (the lower limit of the reference range) 100% specificity in healthy volunteers and homozygous for FV Leiden mutation; high specificity in subjects heterozygous for FV Leiden mutation (94.3%) or using oral contraception (86.7%); and low specificity in pregnant women (57.1%) was observed. At a cut-off value of 86 IU/dL (the highest protein S value found in heterozygous carriers of mutations in PROS1, a decrease in specificity in all groups was observed.

Total protein S assay from Corgenix (PA)

At a cut-off value of 50 IU/dL (the lower limit of the reference range) 100% specificity in all study groups except pregnant women (65.7% specificity) was observed. At a cut-off value of 45 IU/dL (the highest protein S value found in heterozygous carriers of mutations in PROS1 specificity in pregnant women increased to 85.7%.

Free protein S assay with reagents obtained from DAKO (FPS)

At a cut-off value of 57 IU/dL (the lower limit of the reference range) 100% specificity in healthy volunteers and subjects homozygous for FV Leiden mutation or using oral contraception; high specificity in subjects heterozygous for FV Leiden mutation (91.4%); and low specificity in pregnant women (17.1%) was observed. At a cut-off value of 30 IU/d (the highest protein S value found in heterozygous carriers of mutations in PROS1, an optimal specificity in pregnant women (100%) was observed.

Free protein S assay from Corgenix (MFPA)

At a cut-off value of 63 IU/dL (the lower limit of the reference range) 100% specificity in healthy volunteers and subjects heterozygous or homozygous for FV Leiden mutation or using oral contraception; and low specificity in pregnant women (14.3%) was observed. At a cut-off value of 51 IU/dL (the highest protein S value found in heterozygous carriers of mutations in PROS1, specificity in pregnant women increased to 51,4%.

Free protein S assay from Roche Diagnostics (Liatest)

At a cut-off value of 66 IU/dL (the lower limit of the reference range) 100% specificity in healthy volunteers; high specificity in subjects, heterozygous for FV Leiden mutation (94.3%), homozygous for FV Leiden mutation (94.4%) or using oral contraception (90.0%); and no specificity in pregnant women was observed. At a cut-off value of 48 IU/dL (the highest protein S value found in heterozygous carriers of mutations in PROS1, specificity in subjects heterozygous for FV Leiden mutation or using oral contraception increased to 100% and in pregnant women increased to 34.3%.

Free protein S assay from Instrumentation Laboratory (HemosIL)
At a cut-off value of 65 IU/dL (the lower limit of the reference range) 100% specificity in healthy volunteers and subjects homozygous for FV Leiden mutation or using oral contraception; high specificity in subjects heterozygous for FV Leiden mutation (94.3%); and almost no specificity (0.1%) in pregnant women was observed. At a cut-off value of 47 IU/dL (the highest protein S value found in heterozygous carriers of mutations in PROS1 specificity increased to 100% (heterozygous for FV Leiden mutation), and 62.9% (pregnant women), respectively.

Protein S activity assay from Roche Diagnostics (STA-CLOT)
At a cut-off value of 71 IU/dL (the lower limit of the reference range) 100% specificity in healthy volunteers; high specificity in subjects heterozygous for FV Leiden mutation (80.0%) or using oral contraception (83.3%), moderate specificity in subjects homozygous for FV Leiden mutation (55.6%); and no specificity in pregnant women was observed. At a cut-off value of 48 IU/dL (the highest protein S value found in heterozygous carriers of mutations in PROS1 specificity increased to 97.1% (heterozygous for FV Leiden mutation), 100% (oral contraception), 94.4% (homozygous for FV Leiden mutation) and 31.4% (pregnant women), respectively.

Protein S activity assay from Siemens (PSact)
At a cut-off value of 66 IU/dL (the lower limit of the reference range) 100% specificity in healthy volunteers; low specificity in women who are using oral contraception (23.3%); and (almost) no specificity in subjects heterozygous or homozygous for FV Leiden mutation, and pregnant women was observed. At a cut-off value of 22 IU/dL (the highest protein S value found in heterozygous carriers of mutations in PROS1, specificity increased to 100% (oral contraception), 68.6% (heterozygous for FV Leiden mutation), 22.2% (homozygous for FV Leiden mutation) and 97.1% (pregnant women), respectively.

Protein S activity assay from Precision Biologic (CLOT S)

At a cut-off value of 57 IU/dL (the lower limit of the reference range) 100% specificity in healthy volunteers; high specificity in subjects with oral contraception (80.0%); moderate specificity in subjects heterozygous for FV Leiden mutation (57.1%) or homozygous FV Leiden mutation (61.1%); and no specificity in pregnant women was observed. At a cut-off value of 30 IU/dL (the highest protein S value found in heterozygous carriers of mutations in PROS1, specificity increased to 100% (oral contraception), 91.4% (heterozygous FV Leiden mutation), 88.9% (homozygous FV Leiden mutation) and 45.7% (pregnant women), respectively.

Table 3. Specificity for the diagnosis of heterozygous carriers of mutations in PROS1 of 9 protein S assays

		Total protein S assays				Free protein S assays								Protein S activity assays					
		TPS		PA		FPS		MFPA		Liatest		HemosIL		STA-CLOT		PSact		CLOT S	
Cut-off (IU/dL)		71*	86#	50*	45#	57*	30#	63*	51#	66*	48#	65*	47#	71*	48#	66*	22#	57*	30#
HV	SP (%)	100	80.9	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
(n=47)	FP (n)	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FVL het	SP (%)	94.3	80.0	100	100	91.4	100	100	100	94.3	100	94.3	100	80.0	97.1	0.03	68.6	57.1	91.4
(n=35)	FP (n)	2	7	0	0	3	0	0	0	2	0	2	0	7	1	34	11	15	3
FVL hom	SP (%)	100	88.9	100	100	100	100	100	100	94.4	94.4	100	100	55.6	94.4	0	22.2	61.1	88.9
(n=18)	FP (n)	0	2	0	0	0	0	0	0	1	1	0	0	8	1	18	14	7	2
OC	SP (%)	86.7	60.0	100	100	100	100	100	100	90.0	100	100	100	83.3	100	23.3	100	80.0	100
(n=30)	FP (n)	4	12	0	0	0	0	0	0	3	0	0	0	5	0	23	0	6	0
Pregnant	SP (%)	57.1	11.4	65.7	85.7	17.1	100	14.3	51.4	0	34.3	0.1	62.9	0	31.4	0.03	97.1	0	45.7
(n=35)	FP (n)	15	31	12	5	28	0	30	17	35	23	33	13	35	24	34	1	35	19

Specificities (SP) and false positive results (FP) of nine protein S assays for diagnosis of hereditary protein S deficiency in control groups consisting of healthy volunteers (HV), subjects with the factor V Leiden mutation (FVL) heterozygous (het) and homozygous (hom), women using oral contraception (OC) and pregnant women (pregnant). Two different cut-off values were used; i.e the mean - 2SD for healthy volunteers (*) and the highest protein S value found in heterozygous carriers of mutations in PROS1, associated with a 100% sensitivity (#). Specificity of total protein S assay with reagents obtained from DAKO (TPS), total protein S assay from Corgenix (PA), free protein S assay with reagents obtained from DAKO (FPS), free protein S assay from Corgenix (MFPA), free protein S assay from Roche Diagnostics (Liatest), free protein S assay from Instrumentation Laboratory (HemosIL), protein S activity assay from Roche Diagnostics (STA-CLOT), protein S activity assay from Siemens (PSact), protein S activity assay from Precision Biologic (CLOT S).

DISCUSSION

In this study, we defined the optimal low cut-off levels of nine different protein S assays to identify subjects, belonging to families of probands who had experienced venous thromboembolism, and having a causative PROS1 mutation.

For each assay, we determined a reference range using healthy volunteers. In addition, we used the upper limit obtained with each test method from the PROS1 mutation carriers as an alternative cut-off value.
Marked differences were noted between results obtained with the nine evaluated protein S assays. The lower limit of the normal reference ranged from 50 IU/dL to 71 IU/dL. The highest protein S value found in heterozygous PROS1 mutation carriers ranged from 22 IU/dL to 86 IU/dl.

Our choice to use different calibrators has probably played a role in the variability between the different protein S assays, despite the fact that all commercial reference plasmas were calibrated against international standards (28). Hence, the use in all assays of one calibrator would have reduced the differences between results of the different assays and might have demonstrated potential systemic errors for one or more assays. However, because in daily clinical practice, almost always commercial calibrators, included in the assay kits of the manufacturers are used, we followed this approach. Furthermore, we think that use of one calibrator with one reference range for all assays instead of different calibrators with different assay-related reference ranges would not have changed our study conclusions essentially.

All PROS1 mutation carriers showed protein S values below the lower limit of the reference range with eight out of nine assays, resulting in a 100% sensitivity. Only the total protein S assay with reagents obtained from DAKO (TPS) showed a lower sensitivity (93%).

As expected, presence of factor V Leiden mutations influenced protein S activity measurements, resulting in moderate (STA-CLOT and CLOT S) to completely absent (PSact) specificities. When considering the assay principle, wherein diluted patient plasma is added to protein S-deficient plasma in the presence of purified APC and factor Va (29), the variable sensitivities of these assays to patients factor V Leiden might be related to different amounts of reagents factor Va (30).

In line with a previous study (31), we found almost normal protein S levels in women using a second generation oral contraception. The PSact activity assay had a lower specificity (23.3%) that could be restored to 100% by lowering the cut-off value to 22 IU/dL.

Because protein S levels steadily decline with gestational age (19-23), we confirmed the occurrence of low total, free, and activity protein S values in our group of healthy women in the third trimester of pregnancy, causing low specificities. The much lower protein S activity levels in this group might be attributed to higher factor VIII levels, as has been reported before (29). Again, when lowering the cut-off levels to values equal to the highest protein S value found in heterozygous carriers of mutations in PROS1, almost all specificities increased considerably.

The strength of our study is that we defined for the first time optimal low cut-off levels of nine different protein S assays to identify subjects with a hereditary protein S deficiency caused by PROS1 mutations. When using cut-off levels equal to the highest protein S value found in the heterozygous carriers of mutations in PROS1, ensuring 100% sensitivity, the specificity of almost all tested assays increases considerably, by which misclassification can be maximally avoided.

Further strengths of this study are the use of a well-defined reference group of protein S-deficient subjects with a genetically-confirmed PROS1 mutation; control subjects with well-defined confounders related to

protein S measurements, i.e. as oral contraception usage, pregnancy and factor V Leiden mutation; the comparison of nine different total, free and activity protein S assays in a large well-controlled set of experiments; and performance of these assays by one and the same technician using one lot of reagents.

Some methodological aspects of our study warrant comments. First, this study indicates that lowering the cut-off values to the highest value obtained from heterozygous carriers of mutations in PROS1 improves the clinical performance of protein S assays. Although this improves the specificity within the confines of this study, it might result in reduced sensitivity for the detection of hereditary protein S deficiency due to other PROS1 mutations with higher protein S values, not found in this study. However, we have recently demonstrated that decreased free protein S levels are a risk factor for venous thrombosis in subjects only with levels below 30 IU/dL (16, 17). Accordingly, in this study, most heterozygous carriers of PROS1 mutations exhibited free protein S levels far below the level of 50 IU/dL, the level that one would expect them to be for a heterozygous deficiency. Approximately 60% of the total amount of protein S forms a 1:1 stoichiometry with the β-chain of complement component C4-binding protein (C4BP) (4). In vivo, all C4BPβ+ isoform molecules circulate bound to protein S. It seems as if lower total protein S levels alter the equilibrium between bound and free protein S in favour of the bound protein S fraction. Such a mechanism could be of use to maintain steady levels of protein S, as has been suggested before (32). In addition, because the dissociation of the C4BP-protein S complex is a time-, dilution- and temperature-dependent process, free protein S measurement may be hampered by certain assay conditions, like, for instance short incubation time (33).

Second, in the present study almost all heterozygous PROS1 carriers showed a type I protein S deficient phenotype, in line with our previous findings showing that only 1% of type III deficient subjects had free protein S levels below 30 IU/dL (16, 17). Due to inclusion of subjects, based on total

and free protein S measurements, we did not include type II protein S deficient subjects in this study.

Third, healthy women tended to present with lower protein S levels than men, which reached statistically significance when using the Liatest and HemosIL free protein S assays, or the STA-CLOT and CLOT S protein S activity assays (data not shown). Although, in line with other studies (5, 20, 34-36), the level of difference between men and woman was always low, one can argue to use gender-related normal reference ranges with these four assays, since lower cut-off values for healthy women might be expected to yield a little increase in specificity. However, no statistically significant gender differences were found for the PROS1 mutation carriers. Therefore the cut-off values, based on the highest protein S value found in the group heterozygous PROS1 mutation carriers, were not different for women and men, indicating that the influence of gender differences on specificity of the evaluated protein S assays is of limited importance in this study.

Finally, the current study, well controlled and performed within a single laboratory, provides valuable data about expected outcomes for various protein S assays comparing true protein S-deficient positives, possible interference from factor V Leiden (potential false positive for protein S activity assays), and other confounders (oral contraceptives, pregnancy). Unfortunately, in daily clinical practice, there are many other confounders (36). For example, preanalytical variables, like collection into inappropriate collection tubes, under-filled collection and delayed processing will lead to greater assay variability and a higher incidence of false positives. In addition, external quality assurance studies have shown that laboratories can use the same test plasma and the same assay kit but still obtain vastly different test results with between-laboratory CVs, ranging from 10 to 40% (36-42). As we did not obtain inter-laboratory variability, this is a limitation, since it would have narrowed the discriminatory range for negative/positive. Finally, previous studies have shown that around a third of submitted samples with low protein S levels also yielded a high PT, suggesting that these samples

were derived from patients on oral anticoagulant therapy (36, 43, 44). Thus, anticoagulant therapy contributes to false positive detection and may also be an important confounder in clinical practice.

In conclusion, eight out of nine protein S assays showed a 100% sensitivity and 100% specificity to detect heterozygous protein S deficiency-associated PROS1 mutation carriers with values far below the lower limit of the reference values obtained from healthy volunteers. When using low cut-off levels equal to the highest protein S value found in the genetically confirmed protein S deficient study group, the specificities in subjects with factor V Leiden mutations or pregnant women increase considerably by which misclassification can be maximally avoided.

REFERENCES

1. Walker FJ. Regulation of activated protein C by a new protein. A possible function for bovine protein S. J Biol Chem. 1980;255:5521-5524.
2. Walker FJ. Regulation of activated protein C by protein S. The role of phospholipid in factor Va inactivation. J Biol Chem. 1981;256:11128-11131.
3. Walker FJ, Chavin SI, Fay PJ. Inactivation of factor VIII by activated protein C and protein S. Arch Biochem Biophys. 1987;252:322-328.
4. Dahlbäck B. The tale of protein S and C4b-binding protein, a story of affection. Thromb Haemost. 2007;98:90-96.
5. Dykes AC, Walker ID, McMahon AD, Islam SI, Tait RC. A study of Protein S antigen levels in 3788 healthy volunteers: influence of age, sex and hormone use, and estimate for prevalence of deficiency state. Br J Haematol. 2001;113:636-41.
6. Seligsohn U, Lubetsky A. Genetic susceptibility to venous thrombosis. N Engl J Med. 2001;344:1222-1231.
7. Schattner A, Kasher I, Berrebi A. Causes and outcome of deep-vein thrombosis in otherwise-healthy patients under 50 years. QJM. 1997;90:283-287.
8. Pabinger I, Brücker S, Kyrle PA, Schneider B, Korninger HC, Niessner H, Lechner K. Hereditary deficiency of antithrombin III, protein C and protein S: prevalence in patients with a history of venous thrombosis and criteria for rational patient screening. Blood Coagul Fibrinolysis. 1992;3:547-53.
9. Heijboer H, Brandjes DP, Büller HR, Sturk A, ten Cate JW. Deficiencies of coagulation-inhibiting and fibrinolytic proteins in outpatients with deep-vein thrombosis. N Engl J Med. 1990;323:1512-6.

10. Gladson CL, Scharrer I, Hach V, Beck KH, Griffin JH. The frequency of type I heterozygous protein S and protein C deficiency in 141 unrelated young patients with venous thrombosis. Thromb Haemost. 1988;59:18-22.

11. Borgel D, Duchemin J, Alhenc-Gelas M, Matheron C, Aiach M, Gandrille S. Molecular basis for protein S hereditary deficiency: genetic defects observed in 118 patients with type I and type IIa deficiencies. The French Network on Molecular Abnormalities Responsible for Protein C and Protein S Deficiencies. J Lab Clin Med. 1996;128:218-27.

12. Faioni EM, Valsecchi C, Palla A, Taioli E, Razzari C, Mannucci PM. Free protein S deficiency is a risk factor for venous thrombosis. Thromb Haemost. 1997;78:1343-6.

13. Makris M, Leach M, Beauchamp NJ, Daly ME, Cooper PC, Hampton KK, Bayliss P, Peake IR, Miller GJ, Preston FE. Genetic analysis, phenotypic diagnosis, and risk of venous thrombosis in families with inherited deficiencies of protein S. Blood. 2000;95:1935-41.

14. Martinelli I, Mannucci PM, De Stefano V, Taioli E, Rossi V, Crosti F, Paciaroni K, Leone G, Faioni EM. Different risks of thrombosis in four coagulation defects associated with inherited thrombophilia: a study of 150 families. Blood. 1998;92:2353-8.

15. Simmonds RE, Ireland H, Lane DA, Zöller B, García de Frutos P, Dahlbäck B. Clarification of the risk for venous thrombosis associated with hereditary protein S deficiency by investigation of a large kindred with a characterized gene defect. Ann Intern Med. 1998;128:8-14.

16. Lijfering WM, Mulder R, ten Kate MK, Veeger NJ, Mulder AB, van der Meer J. Clinical relevance of decreased free protein S levels: results from a retrospective family cohort study involving 1143 relatives. Blood. 2009;113:1225-30.

17. Brouwer JL, Veeger NJ, van der Schaaf W, Kluin-Nelemans HC, van der Meer J. Difference in absolute risk of venous and arterial thrombosis between familial protein S deficiency type I and type III. Results from a family cohort study to assess the clinical impact of a laboratory test-based classification. Br J Haematol. 2005;128:703-10.

18. Boerger LM, Morris PC, Thurnau GR, Esmon CT, Comp PC. Oral contraceptives and gender affect protein S status. Blood. 1987;69:692-4.

19. Comp PC, Thurnau GR, Welsh J, Esmon CT. Functional and immunologic protein S levels are decreased during pregnancy. Blood. 1986;68:881-5.

20. Henkens CM, Bom VJ, van der Schaaf W, Pelsma PM, Sibinga CT, de Kam PJ, van der Meer J. Plasma levels of protein S, protein C, and factor X: effects of sex, hormonal state and age. Thromb Haemost. 1995;74:1271-5.

21. Malm J, Laurell M, Dahlbäck B. Changes in the plasma levels of vitamin K-dependent proteins C and S and of C4b-binding protein during pregnancy and oral contraception. Br J Haematol. 1988;68:437-443.

22. Said JM, Ignjatovic V, Monagle PT, Walker SP, Higgins JR, Brennecke SP. Altered reference ranges for protein C and protein S during early pregnancy: Implications for the diagnosis of protein C and protein S deficiency during pregnancy. Thromb Haemost. 2010;103:984-8.

23. Szecsi PB, Jørgensen M, Klajnbard A, Andersen MR, Colov NP, Stender S. Haemostatic reference intervals in pregnancy. Thromb Haemost. 2010;103:718-27.

24. Faioni EM, Franchi F, Asti D, Sacchi E, Bernardi F, Mannucci PM. Resistance to activated protein C in nine thrombophilic families: interference in a protein S functional assay. Thromb Haemost. 1993;70:1067-71.

25. Faioni EM, Boyer-Neumann C, Franchi F, Wolf M, Meyer D, Mannucci PM. Another protein S functional assay is sensitive to resistance to activated protein C. Thromb Haemost. 1994;72:648.

26. Ten Kate MK, Platteel M, Mulder R, Terpstra P, Nicolaes GA, Reitsma PH, van der Steege G, van der Meer J. PROS1 analysis in 87 pedigrees with hereditary protein S deficiency demonstrates striking genotype-phenotype associations. Hum Mutat. 2008;29:939-47.

27. Sanders Sevall J. Factor V Leiden genotyping using real-time fluorescent polymerase chain reaction. Mol Cell Probes. 2000;14:249-253.

28. Hubbard AR. Standardisation of protein S in plasma: calibration of the 1st International Standard. Thromb Haemost. 1997;78:1237-1241.

29. Goodwin AJ, Rosendaal FR, Kottke-Marchant K, Bovill EG. A review of the technical, diagnostic, and epidemiologic considerations for protein S assays. Arch Pathol Lab Med. 2002;126:1349-66.

30. Boyer-Neumann C, Bertina RM, Tripodi A, D'Angelo A, Wolf M, Vigano D'Angelo S, Mannucci PM, Meyer D, Larrieu MJ. Comparison of functional assays for protein S: European collaborative study of patients with congenital and acquired deficiency. Thromb Haemost. 1993;70:946-50.

31. Tans G, Curvers J, Middeldorp S, Thomassen MC, Meijers JC, Prins MH, Bouma BN, Büller HR, Rosing J. A randomized cross-over study on the effects of levonorgestrel- and desogestrel-containing oral contraceptives on the anticoagulant pathways. Thromb Haemost. 2000;84:15-21.

32. Almasy L, Soria JM, Souto JC, Coll I, Bacq D, Faure A, Mateo J, Borrell M, Muñoz X, Sala N, Stone WH, Lathrop M, Fontcuberta J, Blangero J; Genetic Analysis of Idiopathic Thrombophilia project. A quantitative trait locus influencing free plasma protein S levels on human chromosome 1q: results from the Genetic Analysis of Idiopathic Thrombophilia (GAIT) project. Arterioscler Thromb Vasc Biol. 2003;23:508-11.

33. Persson KE, Hillarp A, Dahlbäck B. Analytical considerations for free protein S assays in protein S deficiency. Thromb Haemost. 2001;86:1144-1147.

34. Sakkinen PA, Cushman M, Psaty BM, Kuller LH, Bajaj SP, Sabharwal AK, Boineau R, Macy E, Tracy RP. Correlates of antithrombin, protein

C, protein S, and TFPI in a healthy elderly cohort. Thromb Haemost. 1998;80:134-9.

35. Lowe GD, Rumley A, Woodward M, Morrison CE, Philippou H, Lane DA, Tunstall-Pedoe H. Epidemiology of coagulation factors, inhibitors and activation markers: the Third Glasgow MONICA Survey. I. Illustrative reference ranges by age, sex and hormone use. Br J Haematol. 1997;97:775-84.

36. Favaloro EJ, Soltani S, McDonald J, Grezchnik E, Easton L. Laboratory identification of familial thrombophilia: do the pitfalls exceed the benefits? A reassessment of ABO-blood group, gender, age, and other laboratory parameters on the potential influence on a diagnosis of protein C, protein S, and antithrombin deficiency and the potential high risk of a false positive diagnosis. Lab Hematol. 2005;11:174-84.

37. Favaloro EJ. Learning from peer assessment: the role of the external quality assurance multilaboratory thrombophilia test process. Semin Thromb Hemost. 2005;31:85-89.

38. Favaloro EJ, Bonar R, Sioufi J, Wheeler M, Low J, Aboud M, Duncan E, Smith J, Exner T, Lloyd J, Marsden K; RCPA QAP in Haematology. Multilaboratory testing of thrombophilia: current and past practice in Australasia as assessed through the Royal College of Pathologists of Australasia Quality Assurance Program for Hematology. Semin Thromb Hemost. 2005;31:49-58.

39. Jennings I, Kitchen S, Woods TA, Preston FE. Multilaboratory testing in thrombophilia through the United Kingdom National External Quality Assessment Scheme (Blood Coagulation) Quality Assurance Program. Semin Thromb Hemost. 2005;31:66-72.

40. Meijer P, Kluft C, Haverkate F, De Maat MP. The long-term within- and between-laboratory variability for assay of antithrombin, and proteins C and S: results derived from the external quality assessment program for thrombophilia screening of the ECAT Foundation. J Thromb Haemost. 2003;1:748-53.

41. Meijer P, Haverkate F. External quality assessment and the laboratory diagnosis of thrombophilia. Semin Thromb Hemost. 2005;31:59-65.

42. Meijer P, Haverkate F, Kluft C. Performance goals for the laboratory testing of antithrombin, protein C and protein S. Thromb Haemost. 2006;96:584-589.

43. Florell SR, Rodgers GM, III. Utilization of testing for activated protein C resistance in a reference laboratory. Am J Clin Pathol. 1996;106:248-252.

44. Johnston AM, Aboud M, Morel-Kopp MC, Coyle L, Ward CM. Use of a functional assay to diagnose protein S deficiency; inappropriate testing yields equivocal results. Intern Med J. 2007;37:409-11.

Chapter 6

PROS1 Heerlen polymorphism is associated with increased free plasma TFPI levels

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Protein S is a vitamin K-dependent glycoprotein that circulates in plasma in both a bound and free form. Approximately 60% of the total plasma amount of protein S forms a 1:1 stoichiometric complex with the ß-chain of complement component C4-binding protein (1). Free plasma protein S functions as a non-enzymatic cofactor for activated protein C in the proteolytic degradation of activated coagulation factors V and VIII (2-4). In addition, protein S stimulates tissue factor pathway inhibitor (TFPI) in the inactivation of factor Xa (5). Most of the TFPI (50-80%) is bound to glycosyl phosphatidyinositol on the endothelial surface, from which it can be partly released by heparin (6-8). TFPI is also present within platelets accounting for about 10% of the TFPI present in whole blood (9). Plasma TFPI is mainly truncated and bound to lipoproteins. Approximately 5% of plasma TFPI is in the free full-length form (10, 11). Deficiencies of protein S (12, 13) as well as low TFPI levels (14-16) are risk factors for development of venous thromboembolism (VTE). More recently, it has been reported that protein S and TFPI levels covariate in normal individuals (17) and plasma TFPI levels are low in patients with a protein S deficiency (18). Moreover, while normalization of the protein S level hardly affected the increased thrombin generation in protein S-deficient plasma, normalization of the TFPI level reduced the thrombin generation, both at low tissue factor concentration (1.5 pM) in absence of activated protein C, and at high tissue factor concentration (10 pM) in presence of activated protein C (18). Simultaneous normalization of both protein S and TFPI levels completely corrected the increased thrombin generation. Therefore, it has been suggested that combined low levels of protein S and TFPI may synergize in causing thrombosis. Previously, we analysed the association between presence of PROS1 mutations and hereditary protein S deficiency in individuals who were selected from earlier family cohort studies and belonged to families with increased VTE (19). Whether or not the protein S Heerlen polymorphism (20) contributes to thrombosis is still under debate. Although this polymorphism is associated with low levels of free protein S (type III deficiency) and has been reported to be more prevalent in groups of thrombosis patients (21, 22), other studies have found no clear correlation between the PROS1

Heerlen polymorphism and increased risk of venous thrombosis (20, 23). To understand the possible lack of thrombotic complications, we investigated the hypothesis that the PROS1 Heerlen polymorphism is associated with increased TFPI plasma levels.

Protein S-deficient study subjects were selected from previous family cohort studies and belonged to families with increased VTE (19). Venous blood samples from 7 subjects with a heterozygous PROS1 Heerlen polymorphism belonging to five different families (PSHeerlen group) and from 19 subjects carrying a heterozygous PROS1 mutation belonging to eight different families (PROS1 mutation group) were used. In both protein S-deficient groups, two subjects had experienced a venous thrombosis and no one used oral anticoagulant, heparin, hormone-replacement treatment or oral contraceptives at the time of blood sampling. Venous blood samples were taken through identical protocols from 17 male and 13 female in house healthy volunteers (HV). Because TFPI levels increase about 1% per year and are lower in females (18) and in patients with a factor V Leiden mutation (15), we evaluated age and sex and excluded subjects with a factor V Leiden mutation in our study (Table 1). Mean age was not significantly different between PSHeerlen (49 years) and HV (44 years), but PROS1 subjects were significantly younger than HV (34 years, $P<0.01$). Females were over-represented among PSHeerlen subjects, although not significantly different from PROS1 and HV subjects. Total and free protein S antigen levels, measured after precipitation of protein S bound to C4BP with 3.75% PEG 6000, were assayed in duplicate with an enzyme-linked immunosorbent assay with reagents obtained from DAKO, Glostrup, Denmark, as previously described (24). Free TFPI antigen plasma levels were assayed in duplicate with a commercial available enzyme-linked immunosorbent assay (Asserachrom Free TFPI, Diagnostica Stago, Asnieres, France). Continuous variables were expressed as means ± standard deviation and categorical data as counts. Differences between groups were evaluated by the Student's t-test for continuous data and by the Fisher's exact test for categorical data. The relationship between protein S and free TFPI levels

were calculated using the Pearson Correlation Coefficient (r). A *P*-value of less than 0.05 indicated statistical significance. Analyses were performed using PASW Statistics, version 18 SPSS Inc.

In line with an earlier report (18), we found decreased free TFPI levels in subjects carrying a heterozygous PROS1 mutation (11 ± 3 ng/mL *vs* 14 ± 3 ng/mL in HV, *P*<0.01, Table 1A). Within this group, eleven subjects had a type I protein S deficiency with significant lower mean free TFPI levels compared to the free TFPI levels found in the eight type III protein S-deficient subjects (10 ng/mL vs 13 ng/mL, *P*<0.05). Additionally, strong positive correlations between free TFPI levels and both total protein S (r=0.50, *P*<0.05) and free protein S (r=0.37, *P*<0.05) levels were observed in PROS1 subjects (Table 1B). Surprisingly, PSHeerlen subjects with low free protein S levels (protein S deficiency type III) demonstrated increased instead of low free TFPI levels (18 ± 3 ng/mL *vs* 14 ± 3 ng/mL in HV, *P*<0.01, Table 1A). Still, a strong correlation was present between free TFPI and free protein S levels in these subjects (r=0.64, *P*<0.05, Table 1B). These higher free TFPI levels in PSHeerlen type III carriers may to some extent be attributed to the higher protein S levels as compared to the low protein S and free TFPI levels in the PROS1 subjects.

Table 1A. Characteristics, total and free protein S and free TFPI

Group	N	VTE (n)	Age (years)	Sex (m/f)	Free TFPI (ng/ml)	Total PS (IU/dL)	Free PS (IU/dL)
Healthy volunteers	30	0	44 ± 11	17/13	14 ± 3	129 ± 30	85 ± 19
PROS1 mutation	19	2	34 ± 15**	11/8	11 ± 3**	60 ± 11**	12 ± 5**
PROS1 Heerlen polymorphism	7	2	49 ± 20	4/7	18 ± 3**	105 ± 17*	52 ± 14**

PS indicates protein S. Continuous variables are expressed as mean ± standard deviation; significant difference *versus* healthy volunteers: * *P*<0.05; ***P*<0.01.

Table 1B. Correlations between total protein S, free protein S and free TFPI levels

Group	N	Total PS vs free PS	Total PS vs free TFPI	Free PS vs free TFPI
Healthy volunteers	30	0.61**	-0.03	0.42*
PROS1 mutation	19	0.03	0.50*	0.37*
PROS1 Heerlen polymorphism	7	0.09	0.48	0.64*

PS indicates protein S. Significance: * $P<0.05$, ** $P<0.01$.

However, this still leaves the question why PSHeerlen carriers exhibit increased free TFPI levels even higher than in healthy controls. Our data suggests that some other, yet unknown mechanism, independent of protein S levels and strong enough to overcome the lowering effect of protein S, is responsible for the additional increase in free TFPI levels in this group. Increased free TFPI may reflect increased endothelial cell-associated TFPI or may be caused by increased release from the endothelial cells or decreased clearance. Increased affinity for its ligands due to decrease of glycosylation at Asn458 of PSHeerlen has been suggested before (25). Thus, one may speculate that the altered glycosylation pattern of PSHeerlen may perhaps contribute to changes in binding affinities of protein S with TFPI, which may result in increased production, more release from the endothelium, and/or decreased clearance of free TFPI. Further investigations are needed to confirm our results and reveal the underlying mechanisms.

Our results are in accordance with other studies showing that TFPI levels are low in subjects with hereditary protein S deficiency type I and correlate with protein S levels (17, 18). However, in contrast to the report by Castoldi et al. (26), we found increased instead of normal free TFPI levels in subjects with protein S deficiency type III associated with the PSHeerlen. Protein S deficiency type III is a heterogeneous category and Castoldi et al. (26) defined protein S deficiency type III on the basis of protein S levels, irrespective of mutational status. The fact that their protein S deficiency type III study group contained only two carriers with the PSHeerlen may explain the normal instead of high TFPI levels in their group. Furthermore, in

our study free TFPI levels were measured, whereas Castoldi et al measured full-length TFPI. Based on firstly other data (14) that showed similar total, free and TFPI activity results and secondly because free TFPI and full-length TFPI largely overlap, we do not expect that we would have found essentially different results by measuring full-length TFPI.

It is tempting to speculate that high free TFPI levels in subjects with the PS Heerlen could compensate for low free protein S levels and may reset the haemostatic balance to a less-thrombotic phenotype.
Some issues warrant further attention. First, the average level of free protein S (12 IU/dL, Table 1A) is lower than expected in our heterozygous PROS1 mutation group. The reason for this is unknown, but may be related to the type of causal mutations and/or free protein S assay conditions (24). Second, in contrast with our results in protein S-deficient subjects and findings of Dahm et al. (17), we did not find a correlation between total protein S and free TFPI levels in healthy volunteers. Perhaps the large variation in total protein S levels together with the relative small number of healthy volunteers in our study may explain the different findings. Essentially, more studies are needed to confirm our data and to further evaluate the role of TFPI in inherited protein S-deficiencies.

In conclusion, we have shown for the first time that in contrast to subjects with a causative PROS1 mutation resulting in low protein S levels and low free TFPI levels, carriers of the PS Heerlen demonstrate increased free TFPI levels. The high free TFPI levels might counteract the prothrombotic effects of low free protein S levels and contribute to a less-thrombotic phenotype in subjects with the PS Heerlen. Although we used a relative small sample size, our findings may have important implications as relative low TFPI levels might identify subjects with protein S deficiency type III with increased risk of thrombosis.

REFERENCES

1. Dahlbäck B. The tale of protein S and C4b-binding protein, a story of affection. Thromb Haemost. 2007;98:90-6.
2. Walker FJ. Regulation of activated protein C by a new protein. A possible function for bovine protein S. J Biol Chem. 1980;255:5521-4.
3. Walker FJ. Regulation of activated protein C by protein S. The role of phospholipid in factor Va inactivation. J Biol Chem. 1981;256:11128-31.
4. Walker FJ, Chavin SI, Fay PJ. Inactivation of factor VIII by activated protein C and protein S. Arch Biochem Biophys. 1987;252:322-8.
5. Hackeng TM, Seré KM, Tans G, Rosing J. Protein S stimulates inhibition of the tissue factor pathway by tissue factor pathway inhibitor. Proc Natl Acad Sci U S A. 2006;103:3106-11.
6. Bajaj MS, Kuppuswamy MN, Saito H, Spitzer SG, Bajaj SP. Cultured normal human hepatocytes do not synthesize lipoprotein-associated coagulation inhibitor: evidence that endothelium is the principal site of its synthesis. Proc Natl Acad Sci U S A. 1990;87:8869-73.
7. Sandset PM, Abildgaard U, Larsen ML. Heparin induces release of extrinsic coagulation pathway inhibitor (EPI). Thromb Res. 1988;50:803-13.
8. Mast AE, Acharya N, Malecha MJ, Hall CL, Dietzen DJ. Characterization of the association of tissue factor pathway inhibitor with human placenta. Arterioscler Thromb Vasc Biol. 2002;22:2099-104.
9. Novotny WF, Girard TJ, Miletich JP, Broze GJ Jr. Platelets secrete a coagulation inhibitor functionally and antigenically similar to the lipoprotein associated coagulation inhibitor. Blood. 1988;72:2020-5.
10. Lindahl AK, Sandset PM, Abildgaard U. The present status of tissue factor pathway inhibitor. Blood Coagul Fibrinolysis. 1992;3:439-49.
11. Broze GJ Jr, Lange GW, Duffin KL, MacPhail L. Heterogeneity of plasma

tissue factor pathway inhibitor. Blood Coagul Fibrinolysis. 1994;5:551-9.

12. Comp PC, Esmon CT. Recurrent venous thromboembolism in patients with a partial deficiency of protein S. N Engl J Med. 1984;311:1525-8.

13. Schwarz HP, Fischer M, Hopmeier P, Batard MA, Griffin JH. Plasma protein S deficiency in familial thrombotic disease. Blood. 1984;64:1297-300.

14. Dahm A, Van Hylckama Vlieg A, Bendz B, Rosendaal F, Bertina RM, Sandset PM. Low levels of tissue factor pathway inhibitor (TFPI) increase the risk of venous thrombosis. Blood. 2003;101:4387-92.

15. Hoke M, Kyrle PA, Minar E, Bialonzcyk C, Hirschl M, Schneider B, Kollars M, Weltermann A, Eichinger S. Tissue factor pathway inhibitor and the risk of recurrent venous thromboembolism. Thromb Haemost. 2005;94:787-90.

16. Zakai NA, Lutsey PL, Folsom AR, Heckbert SR, Cushman M. Total tissue factor pathway inhibitor and venous thrombosis. The Longitudinal Investigation of Thromboembolism Etiology. Thromb Haemost. 2010;104:207-12.

17. Dahm AE, Sandset PM, Rosendaal FR. The association between protein S levels and anticoagulant activity of tissue factor pathway inhibitor type 1. J Thromb Haemost. 2008;6:393-5.

18. Castoldi E, Simioni P, Tormene D, Rosing J, Hackeng TM. Hereditary and acquired protein S deficiencies are associated with low TFPI levels in plasma. J Thromb Haemost. 2010;8:294-300.

19. Ten Kate MK, Platteel M, Mulder R, Terpstra P, Nicolaes GA, Reitsma PH, van der Steege G, van der Meer J. PROS1 analysis in 87 pedigrees with hereditary protein S deficiency demonstrates striking genotype-phenotype associations. Hum Mutat. 2008;29:939-47.

20. Bertina RM, Ploos van Amstel HK, van Wijngaarden A, Coenen J, Leemhuis MP, Deutz-Terlouw PP, van der Linden IK, Reitsma PH. Heerlen polymorphism of protein S, an immunologic polymorphism due to

dimorphism of residue 460. Blood. 1990;76:538-48.

21. Duchemin J, Gandrille S, Borgel D, Feurgard P, Alhenc-Gelas M, Matheron C, Dreyfus M, Dupuy E, Juhan-Vague I, Aiach M. The Ser 460 to Pro substitution of the protein S alpha (PROS1) gene is a frequent mutation associated with free protein S (type IIa) deficiency. Blood. 1995;86:3436-43.

22. Espinosa-Parrilla Y, Navarro G, Morell M, Abella E, Estivill X, Sala N. Homozygosity for the protein S Heerlen allele is associated with type I PS deficiency in a thrombophilic pedigree with multiple risk factors. Thromb Haemost. 2000;83:102-6.

23. Koenen RR, Gomes L, Tans G, Rosing J, Hackeng TM. The Ser460Pro mutation in recombinant protein S Heerlen does not affect its APC-cofactor and APC-independent anticoagulant activities. Thromb Haemost. 2004;91:1105-14.

24. Mulder R, Ten Kate MK, Kluin-Nelemans HC, Mulder AB. Low cut-off values increase diagnostic performance of protein S assays. Thromb Haemost. 2010;104:618-25.

25. Nicolaes GA, Hackeng TM, Segers K, Rosing J. A structural model of the SHBG domain of human variant protein S Heerlen. Thromb Haemost. 2006;96:538-40.

26. Castoldi E, Maurissen LF, Tormene D, Spiezia L, Gavasso S, Radu C, Hackeng TM, Rosing J, Simioni P. Similar hypercoagulable state and thrombosis risk in type I and type III protein S-deficient individuals from families with mixed type I/III protein S deficiency. Haematologica. 2010;95:1563-71.

Chapter 7

Summary

Normal blood clotting activity requires a delicate balance between procoagulant and anticoagulant factors. A lack of anticoagulant factors can shift this balance towards a hypercoagulant state. Protein S deficiency is considered a risk factor for venous thrombosis. The prevalence of hereditary protein S deficiency ranges from 0.03 to 0.13% in the community to 1-13% in thrombophilic patients. Protein S functions as cofactor for both activated protein C in the proteolytic degradation of cofactors Va and VIIIa, and TFPI in the inhibition of factor Xa. According to the protein S plasma levels, protein S deficiency can be classified in three types; type I with decreased total and free protein S antigen levels; type II, also known as functional defect, with decreased cofactor activity; and type III with decreased free protein S antigen levels, while total protein S antigen levels remain within normal range. This classification may seem straightforward, but the thrombotic risk associated with the different types of protein S deficiency is far from clear. As a result of overlapping protein S levels in carriers and non-carriers of protein S gene (PROS1) mutations, and variation related to age, sex, hormonal state, and several acquired conditions, subjects can easily be misclassified. Moreover, assays suffer from low specificity due to interference with elevated factor VIII levels and factor V Leiden, causing falsely low protein S levels.

The purpose of this thesis is to determine the diagnostic criteria for clinical relevant hereditary and acquired protein S deficiency to identify subjects at risk of first and recurrent venous thrombosis, arterial thrombosis.

Chapter 1 presents a perspective of current knowledge of hemostasis and its role of the coagulation inhibitor protein S.

Chapter 2 describes a single-center retrospective analysis in a thrombophilic family cohort, to define the optimal free protein S cut-off level for detecting subjects who are at risk for venous thrombosis. Only relatives with free protein S levels less than the 5th percentile (< 41 IU/dL) were at risk of first venous thrombosis with an annual incidence of 1.20% (95% confidence

interval [CI], 0.72-1.87) and a recurrence rate of 12.12% (95% CI, 5.23-23.88) per year. Thus, low free protein S levels can identify subjects at risk for venous thrombosis in thrombophilic families, although the cut-off level lies far below the normal range in healthy volunteers.

In **chapter 3** we describe a case-control study showing that cases with free protein S levels less than 41 IU/dL seem to have an increased risk of venous thrombosis during the acute setting, resulting in an odds ratio of 3.4 (95%CI, 0.6-18.9). Adjustments for age, sex, preceding episodes of infectious signs did not change the risk. However, when infectious symptoms were replaced by C-reactive protein levels in the multivariate analysis, the odds ratio of venous thrombosis declined to 1.9 (95%CI, 0.3-11.5). Finally, after adjustment for total C4BP, the odds ratio did not change significantly (OR 3.9, 95%CI, 0.7-23.1). These results suggest that this risk is mediated in part by the acute phase response, but may also be partially genetically determined because the risk of venous thrombosis did not decrease towards 1.0 after correction for the confounders.

Chapter 4 evaluates the risk of low free protein S levels (< 65 IU/dL) and high factor (F) VIII levels (> 150 IU/dL) for arterial thrombosis in a retrospective family cohort study. Annual incidence in relatives with low free protein S levels was 0.26% (95%CI, 0.16-0.40), compared to 0.14% (95%CI, 0.10-0.20) in relatives with normal free protein S levels. In relatives with high FVIII levels, this risk was 0.29% (95%CI, 0.22-0.38) compared to 0.13% (95%CI, 0.09-0.19) in relatives with normal FVIII levels. Mean FVIII levels adjusted for age and sex were respectively 11 IU/dL, 18 IU/dL, and 21 IU/dL higher in relatives with hypertension, diabetes mellitus, and obesity as compared to relatives without these arterial thrombotic risk factors. Indeed, a dose response relation between increasing FVIII levels and body mass index was found. None of these associations were shown for free protein S suggesting that low free protein S levels are more genetically determined in this study.

In **chapter 5** we evaluated nine different protein S assays (2 total protein S antigen assays, 4 free protein S antigen assays, and 3 functional protein S assays) in genetically confirmed protein S-deficient subjects and several control groups (healthy volunteers, oral contraceptive users, pregnant women, and homozygous and heterozygous carriers of the factor V Leiden mutation). With all nine assays, we found significantly lower protein S levels in genetically confirmed protein S-deficient subjects. Eight out of nine protein S assays showed a 100% sensitivity and 100% specificity to detect heterozygous carriers of mutations in PROS1 with values far below the lower limit of the reference values obtained from healthy volunteers. Low specificities were found in subjects with a factor V Leiden mutation and in pregnant women. At lower cut-off levels, equal to the highest protein S value found in heterozygous carriers of mutations in PROS1, the specificity considerably increased in these subjects.

In conclusion, when using low cut-off levels equal to the highest protein S value found in heterozygous carriers of mutations in PROS1, ensuring 100% sensitivity, the specificity in all study groups increased considerably, by which misclassification could be maximally avoided.

Whether or not the protein S Heerlen variant with decreased free protein S levels (type III protein S deficiency) contributes to thrombosis is still under debate. To understand the possible lack of thrombotic complications in this type III protein S deficiency, we investigated in **chapter 6** the hypothesis that subjects with a protein S Heerlen variant have increased TFPI plasma levels. Strong positive correlations between free plasma TFPI levels and both total protein S ($r=0.50$, $P<0.05$) and free protein S ($r=0.37$, $P<0.05$) levels were observed in a control group of subjects with other causative PROS1 mutations. Confirming our hypothesis, subjects carrying the protein S Heerlen variant and having low free protein S levels (protein S deficiency type III) demonstrated increased instead of low free plasma TFPI levels (18 ± 3 ng/mL *vs* 14 ± 3 ng/mL in healthy volunteers, $P<0.01$). Possibly, high free TFPI levels observed in subjects with the PROS1 Heerlen variant might compensate for low free protein S levels and thereby reduce the thrombotic risk.

Chapter 8

Discussion and future perspectives

Among the thrombophilic defects, protein S deficiency is the most difficult defect to study, which complicates the identification of patients who are at risk of thrombosis due to protein S deficiency. In this context, we performed several studies to determine reliable risk estimates and accurate detection values.

Laboratory perspectives

Hereditary protein S deficiency is an autosomal dominant disorder. Over the past 20 years the molecular background of protein S deficiency has become better understood. As a result, more than 200 mutations have been detected, of which missense mutations are the predominant form. However, molecular analysis is not routinely performed. Therefore, we need to relay on surrogate markers for this genetic defect by assessing the laboratory phenotype. This creates many uncertainties. Importantly, protein S levels are influenced by many factors, including age, sex, hormonal state, and several acquired conditions. A further concern relates to the laboratory evaluation (1-7). In general, there are two types of protein S assays, i.e., functional assays and immunological assays. Functional assays measure the cofactor activity of protein S for activated protein C (APC) and can be based on modified partial thromboplastin time (APTT) or prothrombin time (PT) format. Immunological assays measure total and free protein S antigen levels. Based on the plasma concentration of total protein S, free protein S, and APC cofactor activity, protein S deficiency is categorized into three subtypes (8). Protein S deficiency type I is characterized by decreased levels of both total and free protein S. Type II is a functional deficiency with normal protein S antigen levels, but reduced APC cofactor activity. Protein S deficiency type III is characterized by normal levels of total protein S, but decreased levels of free protein S.

Theoretically, functional assays are favoured upon immunological assays as they are able to detect all subtypes. Unfortunately, the assays suffer from low specificity mainly due to the interference by the presence of factor V Leiden mutation and high factor VIII levels (> 250 IU/dL), resulting in falsely

low protein S levels (9). Immunological assays enable the classification of quantitative defects (type I and III). However, both phenotypes have been observed within the same families, suggesting phenotypic variability due to age-dependent increase of total protein S levels causing a shift from type I to type III. In addition, free protein S assays may suffer from poor reproducibility and demonstrate a time-, temperature-, and dilution-dependent increase in free protein S antigen levels (10).

Despite these practical problems, hereditary protein S deficiency is diagnosed based on repeated low protein S levels below the lower limit of a reference range based on healthy volunteers. According to our study (**chapter 5**), most if not all misclassifications due to technical or acquired conditions can be avoided when using the highest value obtained from heterozygous PROS1 mutation carriers, ensuring 100% sensitivity. Therefore, these results emphasize the need to re-evaluate the detection values used for each protein S assay in order to better discriminate between carriers and non-carriers of a PROS1 mutation. Although this improves the specificity within the confines of our study, it might result in reduced sensitivity for the detection of hereditary protein S deficiency in carriers with other PROS1 mutations in other studies (11). Moreover, low protein S levels obtained from functional assays should always be interpreted with caution due to the negative influence of factor V Leiden mutation and high factor VIII levels. Obviously, preanalytical variables like blood collection into inappropriate collection tubes, underfilled tubes, and delayed processing will lead to greater assay variability (12-18). Finally, it is important to realize that many of the samples sent to the laboratory will come from patients on oral anticoagulant therapy, resulting in low protein S levels (12, 19, 20).

Clinical perspectives

Although hereditary protein S deficiency is relatively uncommon in the general population with a prevalence ranging from 0.03% to 0.13%, it is found in 1-13% of thrombophilic families (21, 22). The reasons for this difference in prevalence is not yet fully understood. On the one hand, the risk may have been overestimated by selection of thrombophilic patients from thrombophilic families, ethnicity and study design. On the other hand, reliable risk estimates have been hampered by the inability to accurately diagnose protein S deficiency. Despite these drawbacks, type I protein S deficiency is an established risk factor for venous thrombosis with an annual incidence of 1.0 to 3.1% (23-26). No such evident association has been observed for type II and III protein S deficiency.

Among the subtypes, type II protein S deficiency is relatively uncommon, which is probably caused by a combination of low frequency of causative mutations in the EGF-domains of PROS1 and low specificity of functional assays. To date, only one study investigated the association between congenital protein S deficiency type II and venous thrombosis and observed an annual incidence of 0.24 (95%CI, 0-0.54), which was not significantly increased due to the absence of events among controls (27).

Conflicting results have been obtained for the risk of venous thrombosis associated with type III protein S deficiency. Most clinical studies on protein S type III deficiency refer to laboratory reference values obtained from healthy volunteers (which in our laboratory are < 65 IU/dL).

In **chapter 2** we showed that this cut-off level is too high to identify subjects at risk for venous thrombosis due to protein S type III deficiency. Rather, we identified a threshold level, both for first venous thrombosis and its recurrence, at free protein S levels lower than 41 IU/dL (< 5th percentile in thrombophilic families). Using this cut-off point increased the absolute risk of first venous thrombosis in our study population from 0.20% per year to 1.20% per year and for recurrence from 4.13% per year to 12.12% per

year. However, low free protein S levels probably involve both acquired and genetic factors.

In **chapter 3** we confirmed that free protein S levels below the 5th percentile could also identify subjects who are at increased risk of venous thrombosis in the acute inflammatory setting. Note, increased risk may partially be explained by the acute phase response, as after adjustment for CRP, the risk decreased from 3.4 to 1.9. Furthermore, we did not observe any influence of increased total C4BP levels in the acute inflammatory setting. Possibly, only the C4BP fraction without a ß-chain is increased due to differential regulations of C4BPα and C4BPß isoforms during the acute phase (28). However, our results should be interpreted with caution because the risk we found was not statistically significant, probably because our sample was relatively small. As our study was performed in a university hospital setting, our results cannot directly be translated to the general population. Further research is required to elucidate the underlying mechanism responsible for the associated risk.

From a genetically point of view, most families with a type III protein S deficiency have no PROS1 mutation or the protein S Heerlen polymorphism (29-31). The protein S Heerlen mutation is characterised by a serine to proline substitution at position 460, due to a T>C transition in exon 13 of the PROS1 gene (29). This substitution occurs in the consensus sequence for the potential N-linked glycosylation of Asn458 (29). As a result, protein S molecules with Heerlen mutation show increased clearance leading to low free protein S levels (32). Clearance of protein S Heerlen variant is reduced when bound to C4BP.

Nonetheless, there is much doubt whether there is an association of low free protein S levels caused by the Heerlen variant and increased risk of venous thrombosis. This raised the question which underlying mechanism(s) could be responsible for the lower risk. Since 5 years another function of protein S is known (33). In the presence of protein S, TFPI inhibits factor

Xa at low tissue factor concentrations (initial phase of coagulation). Low levels of free and total TFPI have been associated with venous thrombosis in the Leiden Thrombophilia Study, which is a large population-based case-control study of 474 patients and 474 controls (34). Moreover, decreased levels of TFPI have also been observed in patients with hereditary protein S deficiency (35), suggesting a possible synergistic effect. Therefore, we hypothesized that the tight interplay between both proteins (protein S and TFPI) may also be important in subject with decreased free protein S caused by the PROS1 Heerlen mutation, and might explain the lower risk of venous thrombosis.

In line with a previous study (35), we found decreased free TFPI levels in subjects carrying other heterozygous PROS1 mutations (**chapter 6**). Surprisingly, subjects with low free PS levels caused by the PROS1 Heerlen mutation demonstrated increased instead of low free TFPI levels (18 ± 3 ng/mL *vs* 14 ± 3 ng/mL in HV, $P<0.01$). Increased TFPI levels may reflect increased release from endothelial cells or increased endothelial cell-associated TFPI, or decreased clearance. The altered glycosylation at Asn458 site of the PROS1 Heerlen variant (36) may perhaps contribute to changes in binding affinities of protein S with TFPI, consequently resulting in increased production, more release from the endothelium, and/or decreased clearance of free TFPI. Until now, almost no information regarding the binding site of protein S for TFPI is available. A recent study demonstrated that the third Kunitz-type domain and, to a lesser extent, the carboxyl-terminus of TFPI are important for the Ca2+ and phospholipid-dependent protein S enhancement of TFPI mediated factor Xa inhibition (37). A single amino acid change in the third Kunitz-type domain substantially reduced this inhibition, suggesting that the third Kunitz-type domain possesses the binding site for protein S. When we compared the sequence of the first short consensus repeat of C4BPβ, which binds to protein S, with that of the third Kunitz-type domain of TFPI, a homology of approximately 40% was found (data not shown). Therefore, it may be likely that the sex-hormone globulin domain of protein S that binds to the first short consensus repeat of

C4BPß is also involved in the interaction with TFPI. It would be interesting to determine whether the Heerlen polymorphism (SHBG domain) could alter the interaction with TFPI. Moreover, further investigations are needed to confirm our results and reveal the underlying mechanisms. However, it is tempting to speculate that high free TFPI levels observed in subjects with the Heerlen variant might compensate for low free protein S levels and thereby resetting the haemostatic balance. These findings may have important implications as relatively lower TFPI levels might identify type III protein S deficient subjects at higher risk of venous thrombosis. In line of these results, it might be interesting to investigate whether the ratio between TFPI and free protein S levels could discriminate between high risk and low risk of venous thrombosis.

While thrombophilic risk factors predispose to venous thrombosis, the influence on arterial thrombosis is less clear (38). Some previous data suggest that low free protein S as well as high factor VIII levels are associated with increased risk of arterial thrombosis (39-43). In **chapter 4**, we studied these relations and evaluated whether they are genetically determined and/or acquired. We found a nearly 2-fold increased risk of arterial thrombosis in both relatives with free protein S levels below the lower limit of the normal reference range and relatives with factor VIII levels above the upper limit of the normal reference range.

The association between high factor VIII levels and arterial thrombosis could in part be ascribed to an age-dependent effect of factor VIII, as after adjustment for age the risk decreased from 2.2 to 1.5. Moreover, we investigated the relation with traditional arterial thrombotic risk factors (hypertension, hyperlipidemia, the presence of diabetes mellitus, smoking and obesity defined as body mass index above 30 kg/m^2). Our data indicated that factor VIII levels were influenced by diabetes mellitus, obesity and hypertension. In contrast, the risk associated with low free protein S levels was not influenced by age, nor by any other traditional arterial thrombotic risk factor.

Interestingly, in our study the free protein S levels associated with increased risk of venous thrombosis seem to have to be lower (below the 5th percentile) than the levels related to increased risk of arterial thrombosis (below 65 IU/dL). It seems as if intermediate low free protein S levels predispose only for increased risk of arterial thrombosis and not for increased risk of venous thrombosis. However, the increase in risk of arterial thrombosis is still relatively low in subjects with low free protein S levels and is detected only in epidemiological studies. Probably, subjects with free protein S levels below the 5th percentile might have higher risk of arterial thrombosis. Therefore, further analysis of our data and more studies are required to determine the exact risk of arterial thrombosis related to free protein S levels.

Although this thesis did not include population-based studies, others have recently shown that there is no association between venous thrombosis and protein S deficiency in the normal population (44), even when the cut-off level was far below the normal range in healthy controls. A reason for this contrasting finding could be that inherited protein S deficiency is too rare in a general population to identify persons at risk of venous thrombosis by use of a protein S measurement only. Protein S testing should therefore not be considered in unselected patients with venous thrombosis.

Conclusion

Although protein S deficiency has been associated with thrombosis, reliable risk estimates have been hampered by the difficulty of correctly diagnosing protein S deficiency. When using low cut-off levels equal to the highest protein S value found in heterozygous carriers of mutations in PROS1, ensuring 100% sensitivity, the specificity of both types of protein S assays (functional and immunological) increases considerably, by which misclassification can be maximally avoided. Note, free protein S assays should be the principle assay for detection of true protein S deficiency. Furthermore, low free protein S levels below the 5th percentile can identify subjects who are at risk of venous and probably also arterial thrombosis. However, the latter still requires further research. Potential clinical implications may be prolonged anticoagulant treatment in cases with free protein S levels below the 5th percentile. Furthermore, prophylaxis should be considered in high risk situations such as trauma, surgery, immobilization, pregnancy and puerperium, as well as discouragement of the use of oral contraceptives. Finally, in cases with a type III protein S deficiency with free protein S levels higher than the 5th percentile, the risk level of venous thrombosis might be attributed to the interplay between TFPI and protein S. It would be challenging to investigate the different regulatory mechanisms responsible for differences in risk. Such future studies, including phenotype-genotype association studies may bring us closer to understanding the pathophysiological processes underneath venous and arterial thrombosis in subjects with protein S deficiency.

REFERENCES

1. Said JM, Ignjatovic V, Monagle PT, Walker SP, Higgins JR, Brennecke SP. Altered reference ranges for protein C and protein S during early pregnancy: Implications for the diagnosis of protein C and protein S deficiency during pregnancy. Thromb Haemost. 2010;103:984-8.
2. Vigano-D'Angelo S, D'Angelo A, Kaufman CE, Jr., Sholer C, Esmon CT, Comp PC. Protein S deficiency occurs in the nephrotic syndrome. Ann Intern Med. 1987;107:42-7.
3. Brouwer JL, Bijl M, Veeger NJ, Kluin-Nelemans HC, van der Meer J. The contribution of inherited and acquired thrombophilic defects, alone or combined with antiphospholipid antibodies, to venous and arterial thromboembolism in patients with systemic lupus erythematosus. Blood. 2004;104:143-8.
4. Lijfering WM, Sprenger HG, Georg RR, van der Meulen PA, van der Meer J. Relationship between progression to AIDS and thrombophilic abnormalities in HIV infection. Clin Chem. 2008;54:1226-33.
5. Henkens CMA, Bom VJJ, van der Schaaf W, Pelsma PM, Sibinga CTS, de Kam PJ, van der Meer J. Plasma-Levels of Protein-S, Protein-C, and Factor-X - Effects of Sex, Hormonal State and Age. Thromb Haemost. 1995;74:1271-5.
6. Boerger LM, Morris PC, Thurnau GR, Esmon CT, Comp PC. Oral contraceptives and gender affect protein S status. Blood. 1987;69:692-4.
7. Favaloro EJ, Soltani S, McDonald J, Grezchnik E, Easton L. Laboratory identification of familial thrombophilia: do the pitfalls exceed the benefits? A reassessment of ABO-blood group, gender, age, and other laboratory parameters on the potential influence on a diagnosis of protein C, protein S, and antithrombin deficiency and the potential high risk of a false positive diagnosis. Lab Hematol. 2005;11:174-84.

8. Bertina RM. Proposal for the nomenclature of protein S deficiency. XXXVIII Annual meeting Scientific and Standardization Committee of the ISTH, Munchen, Germany, 1992.

9. Goodwin AJ, Rosendaal FR, Kottke-Marchant K, Bovill EG. A review of the technical, diagnostic, and epidemiologic considerations for protein S assays. Arch Pathol Lab Med. 2002;126:1349-66.

10. Persson KE, Hillarp A, Dahlbäck B. Analytical considerations for free protein S assays in protein S deficiency. Thromb Haemost. 2001;86:1144-7.

11. Biguzzi E, Razzari C, Lane DA, Castaman G, Cappellari A, Bucciarelli P, Fontana G, Margaglione M, D'Andrea G, Simmonds RE, Rezende SM, Preston R, Prisco D, Faioni EM; Protein S Italian Team. Molecular diversity and thrombotic risk in protein S deficiency: the PROSIT study. Hum Mutat. 2005;25:259-69.

12. Favaloro EJ, Soltani S, McDonald J, Grezchnik E, Easton L. Laboratory identification of familial thrombophilia: do the pitfalls exceed the benefits? A reassessment of ABO-blood group, gender, age, and other laboratory parameters on the potential influence on a diagnosis of protein C, protein S, and antithrombin deficiency and the potential high risk of a false positive diagnosis. Lab Hematol. 2005;11:174-84.

13. Favaloro EJ. Learning from peer assessment: the role of the external quality assurance multilaboratory thrombophilia test process. Semin Thromb Hemost. 2005;31:85-89.

14. Favaloro EJ, Bonar R, Sioufi J, Wheeler M, Low J, Aboud M, Duncan E, Smith J, Exner T, Lloyd J, Marsden K; RCPA QAP in Haematology. Multilaboratory testing of thrombophilia: current and past practice in Australasia as assessed through the Royal College of Pathologists of Australasia Quality Assurance Program for Hematology. Semin Thromb Hemost. 2005;31:49-58.

15. Jennings I, Kitchen S, Woods TA, Preston FE. Multilaboratory testing in thrombophilia through the United Kingdom National External Quality Assessment Scheme (Blood Coagulation) Quality Assurance Program. Semin Thromb Hemost. 2005;31:66-72.

16. Meijer P, Kluft C, Haverkate F, De Maat MP. The long-term within- and between-laboratory variability for assay of antithrombin, and proteins C and S: results derived from the external quality assessment program for thrombophilia screening of the ECAT Foundation. J Thromb Haemost. 2003;1:748-53.

17. Meijer P, Haverkate F. External quality assessment and the laboratory diagnosis of thrombophilia. Semin Thromb Hemost. 2005;31:59-65.

18. Meijer P, Haverkate F, Kluft C. Performance goals for the laboratory testing of antithrombin, protein C and protein S. Thromb Haemost. 2006;96:584-589.

19. Florell SR, Rodgers GM, III. Utilization of testing for activated protein C resistance in a reference laboratory. Am J Clin Pathol. 1996;106:248-252.

20. Johnston AM, Aboud M, Morel-Kopp MC, Coyle L, Ward CM. Use of a functional assay to diagnose protein S deficiency; inappropriate testing yields equivocal results. Intern Med J. 2007;37:409-11.

21. Dykes AC, Walker ID, McMahon AD, Islam SIAM, Tait RC. A study of Protein S antigen levels in 3788 healthy volunteers: influence of age, sex and hormone use, and estimate for prevalence of deficiency state. Br J Haematol. 2001;113:636-41.

22. Seligsohn U, Lubetsky A. Genetic susceptibility to venous thrombosis. N Engl J Med. 2001;344:1222-31.

23. Schattner A, Kasher I, Berrebi A. Causes and outcome of deep-vein thrombosis in otherwise-healthy patients under 50 years. QJM. 1997;90:283-287.

24. Pabinger I, Brücker S, Kyrle PA, Schneider B, Korninger HC, Niessner

H, Lechner K. Hereditary deficiency of antithrombin III, protein C and protein S: prevalence in patients with a history of venous thrombosis and criteria for rational patient screening. Blood Coagul Fibrinolysis. 1992;3:547-53.

25. Heijboer H, Brandjes DP, Büller HR, Sturk A, ten Cate JW. Deficiencies of coagulation-inhibiting and fibrinolytic proteins in outpatients with deep-vein thrombosis. N Engl J Med. 1990;323:1512-6.

26. Gladson CL, Scharrer I, Hach V, Beck KH, Griffin JH. The frequency of type I heterozygous protein S and protein C deficiency in 141 unrelated young patients with venous thrombosis. Thromb Haemost. 1988;59:18-22.

27. Alhenc-Gelas M, Canonico M, Morange PE, Emmerich J; Geht Genetic Thrombophilia Group. Protein S inherited qualitative deficiency: novel mutations and phenotypic influence. J Thromb Haemost. 2010;8:2718-26.

28. García de Frutos P, Alim RI, Härdig Y, Zöller B, Dahlbäck B. Differential regulation of alpha and beta chains of C4b-binding protein during acute-phase response resulting in stable plasma levels of free anticoagulant protein S. Blood. 1994;84:815-22.

29. Bertina RM, Ploos van Amstel HK, van Wijngaarden A, Coenen J, Leemhuis MP, Deutz-Terlouw PP, van der Linden IK, Reitsma PH. Heerlen polymorphism of protein S, an immunologic polymorphism due to dimorphism of residue 460. Blood. 1990;76:538-48.

30. Duchemin J, Gandrille S, Borgel D, Feurgard P, Alhenc-Gelas M, Matheron C, Dreyfus M, Dupuy E, Juhan-Vague I, Aiach M. The Ser 460 to Pro substitution of the protein S alpha (PROS1) gene is a frequent mutation associated with free protein S (type IIa) deficiency. Blood. 1995;86:3436-43.

31. Espinosa-Parrilla Y, Navarro G, Morell M, Abella E, Estivill X, Sala N. Homozygosity for the protein S Heerlen allele is associated with type I PS deficiency in a thrombophilic pedigree with multiple risk factors.

Thromb Haemost. 2000;83:102-6.

32. Denis CV, Roberts SJ, Hackeng TM, Lenting PJ. In vivo clearance of human protein S in a mouse model: influence of C4b-binding protein and the Heerlen polymorphism. Arterioscler Thromb Vasc Biol. 2005;25:2209-15.

33. Hackeng TM, Seré KM, Tans G, Rosing J. Protein S stimulates inhibition of the tissue factor pathway by tissue factor pathway inhibitor. Proc Natl Acad Sci U S A. 2006;103:3106-11.

34. Dahm A, Van Hylckama Vlieg A, Bendz B, et al. Low levels of tissue factor pathway inhibitor (TFPI) increase the risk of venous thrombosis. Blood. 2003;101:4387-92.

35. Castoldi E, Simioni P, Tormene D, Rosing J, Hackeng TM. Hereditary and acquired protein S deficiencies are associated with low TFPI levels in plasma. J Thromb Haemost. 2010;8:294-300.

36. Nicolaes GA, Hackeng TM, Segers K, Rosing J. A structural model of the SHBG domain of human variant protein S Heerlen. Thromb Haemost. 2006;96:538-40.

37. Ndonwi M, Tuley EA, Broze GJ Jr. The Kunitz-3 domain of TFPI-alpha is required for protein S-dependent enhancement of factor Xa inhibition. Blood. 2010;116:1344-51.

38. Boekholdt SM, Kramer MH. Arterial thrombosis and the role of thrombophilia. Semin Thromb Hemost. 2007;33:588-96.

39. Romano N, Prosperi V, Basili G, Lorenzetti L, Gentile V, Luceretti R, Biondi G, Goletti O. Acute thrombosis of the superior mesenteric artery in a 39-year-old woman with protein-S deficiency: a case report. J Med Case Reports. 2011;5:17.

40. Archer KA, Lembo T Jr, Haber JA. Protein S deficiency and lower-extremity arterial thrombosis: complicating a common presentation. J Am Podiatr Med Assoc. 2007;97:151-5.

41. Zimmerman AA, Watson RS, Williams JK. Protein S deficiency presenting

as an acute postoperative arterial thrombosis in a four-year-old child. Anesth Analg. 1999;88:535-7.

42. Green D, Otoya J, Oriba H, Rovner R. Protein S deficiency in middle-aged women with stroke. Neurology. 1992;42:1029-33.

43. Bank I, Libourel EJ, Middeldorp S, Hamulyák K, van Pampus EC, Koopman MM, Prins MH, van der Meer J, Büller HR. Elevated levels of FVIII:C within families are associated with an increased risk for venous and arterial thrombosis. J Thromb Haemost. 2005;3:79-84.

44. Ribeiro DD, Pintao MCT, Lijfering WM, Reitsma PH, Rosendaal FR (dec 2011, abstract 538). Free and Total Protein S Antigen Levels on the Risk of Venous Thrombosis: Results From the MEGA Study. Oral and Poster session presented at the 53rd annual meeting of the American Society of Hematology, San Diego, California.

Chapter 9

Nederlandse samenvatting

Voor bloedstelping (hemostase) is fibrinevorming nodig. Fibrinevorming is een complex proces van interacties tussen veel stollingsfactoren. Onder normale fysiologische omstandigheden bestaat er een zeer nauw gereguleerde balans tussen invloeden van factoren die de bloedstolling bevorderen (procoagulante factoren) en factoren die de stolling remmen (anticoagulante factoren). Door een tekort (deficiëntie) aan anticoagulante factoren kan het evenwicht verschuiven in de richting van toegenomen stolling, met als gevolg een verhoogd risico op trombose (trombofilie). Eén van de belangrijkste anticoagulante factoren is proteïne S. Proteïne S wordt voornamelijk gesynthetiseerd door de lever en heeft daarvoor onder andere vitamine K nodig.

Proteïne S is voor ongeveer twee derde deel in het plasma gebonden aan een complementeiwit, het zogenaamde 'complement-C4-binding-protein' (C4BP). Het resterende ongebonden proteïne S is vrij in het plasma aanwezig (vrij proteïne S) en vormt de belangrijkste bron van activiteit. Proteïne S ondersteunt als cofactor de werking van een andere anticoagulante factor, het zogenaamde proteïne C. Het complex tussen proteïne S en geactiveerd proteïne C is in staat geactiveerde stollingsfactoren Va en VIIIa weer te inactiveren en daardoor de fibrinevorming te verlagen. Meer recent is gebleken dat proteïne S de functie van nog een andere anticoagulante factor, de zogenaamde 'Tissue Factor Pathway Inhibitor (TFPI)' ondersteunt, waarbij geactiveerd factor Xa wordt geremd.

Erfelijk bepaalde proteïne S deficiëntie is een weinig voorkomende afwijking met een prevalentie in de algehele bevolking van 0.03 tot 0.13%. Proteïne S deficiëntie leidt tot een familiaire trombofilie met verhoogde kans op het ontstaan van een trombosebeen of een longembolie. Proteïne S deficiëntie komt voor bij 1 tot 13% van de mensen met een familiaire trombofilie. De relatie tussen een verlaagde spiegel totaal of vrij proteïne S en het risico op het optreden van een trombose is niet eenduidig. Plasmaspiegels van proteïne S worden bepaald door een groot aantal factoren, zoals leeftijd, geslacht, hormonale huishouding en aanwezigheid van vele andere

ziekten. Ook het meten van proteïne S concentraties in plasma is complex en wordt beïnvloed door vele andere factoren, zoals onder andere de plasmaconcentraties van C4BP, factor V en factor VIII.

Dit proefschrift beschrijft een aantal studies waarin de relatie tussen de spiegels van verlaagd vrij proteïne S en verschillende vormen van familiaire proteïne S deficiëntie, verworven proteïne S deficiëntie en arteriële trombose zijn onderzocht. Getracht is een optimale afkapwaarde voor het vaststellen van een proteïne S deficiëntie te definiëren.

Hoofdstuk 1 geeft een algeheel overzicht van de huidige inzichten in het proces van hemostase, met speciale aandacht voor de functies van proteïne S.

Hoofdstuk 2 beschrijft een retrospectieve studie, waarin de relatie tussen een verlaagde concentratie vrij proteïne S en het risico op het optreden van een eerste en van een recidief veneuze trombose is onderzocht. Uit de resultaten blijkt dat vrije proteïne S spiegels ver beneden de ondergrens van het normaalwaardengebied, namelijk lager dan het 5^{e} percentiel, pas een verhoogde kans op een eerste of recidief veneuze trombose geven. Door gebruik te maken van deze lage afkapwaarde in plaats van de ondergrens van het normaalwaardengebied, neemt het risico op het optreden van een eerste veneuze trombose bij verlaagde proteïne S spiegels toe van 0.20% (95% BI, 0.11-0.32) per jaar naar 1.20% (95% BI, 0.72-1.87) en voor recidief veneuze trombose van 4.13% (95% BI, 1.34-9.64) per jaar naar 12.12% (95% BI, 5.23-23.88).

Hoofdstuk 3 beschrijft een case-control studie waaruit blijkt dat spiegels van vrij proteïne S beneden het 5^{e} percentiel ook een verhoogd risico op veneuze trombose geven ten tijde van een acute fase reactie, met een odds ratio van 3.4 (95%CI, 0.6-18.9). Na correctie voor leeftijd, geslacht, voorgaande episodes van infectieuze symptomen en C4BP spiegels, bleef het risico onveranderd. Echter, wanneer er gecorrigeerd werd voor CRP spiegels in een multivariate analyse, daalde de odds ratio naar 1.9 (95%CI,

0.3-11.5). De resultaten suggereren, dat het risico op een veneuze trombose tijdens een acute fase deels beïnvloed wordt door de acute fase reactie zelf en deels door een genetische achtergrond.

Hoofdstuk 4 beschrijft de resultaten van een retrospectieve studie waaruit blijkt dat zowel verlaagde vrij proteïne S spiegels (< 65 IU/dL) als verhoogde factor VIII spiegels (>150 IU/dL), gepaard gaan met een tweevoudig verhoogd risico op arteriële trombose. Verhoogde factor VIII spiegels blijken samen te hangen met leeftijd, de klassieke arteriële risicofactoren zoals hoge bloeddruk, hyperlipidemie, diabetes mellitus, roken en obesitas. Factor VIII blijkt zelfs een dose-response relatie met de body-mass-index te hebben. De verlaagde vrij proteïne S spiegels lijken echter niet met leeftijd of klassieke arteriële risicofactoren samen te hangen, suggererend dat erfelijk bepaalde lage proteïne S spiegels zelf een licht verhoogd risico op arteriële trombose geven.

Hoofdstuk 5 richt zich op de techniek van de proteïne S bepalingen en beschrijft een studie, waarin 9 verschillende proteïne S laboratoriumbepalingen (2 totaal proteïne S antigeen testen, 4 vrij proteïne S testen, en 3 functionele proteïne S testen) worden geëvalueerd. Naast plasma van individuen met een genetisch bevestigde proteïne S deficiëntie werd plasma van gezonde vrijwilligers, vrouwen die orale anticonceptie gebruikten, zwangeren en dragers van een homozygote of heterozygote factor V Leiden mutatie, als controle gebruikt. Met alle 9 testen werd bij individuen met een genetisch bevestigde proteïne S deficiëntie een verlaagde proteïne S spiegel gevonden, met voor 8 testen 100% sensitiviteit en voor alle 9 testen 100% specificiteit. Een duidelijk lagere specificiteit werd gevonden bij zwangeren en bij dragers van de factor V Leiden mutatie. Door de hoogst gemeten proteïne S spiegel uit de groep met een genetisch bevestigde proteïne S deficiëntie als afkapwaarde te nemen, verbeterde de specificiteit van alle testen en verkleinde daarmee de kans op vals positieve uitslagen, bij met name zwangeren en dragers van de factor V Leiden mutatie.

Individuen met een proteïne S Heerlen mutatie hebben naast laag-normale totaal proteïne S spiegels verlaagde vrij proteïne S concentraties, waarbij het erop lijkt dat deze variant een lager risico op veneuze trombose geeft dan veel andere proteïne S mutaties. In **hoofdstuk 6** werd de hypothese getoetst dat individuen met een proteïne S Heerlen variant mogelijk een verhoogde TFPI concentratie hebben. Inderdaad werd in de groep personen met een proteïne S Heerlen variant verhoogde TFPI plasma spiegels gevonden (18 ± 3 ng/mL versus 14 ± 3 ng/ml in gezonde vrijwilligers, $P<0.01$). Mogelijk compenseert een hoge TFPI spiegel in personen met een proteïne S Heerlen variant de verlaagde vrij proteïne S concentratie en ontstaat daardoor een minder hoog risico op veneuze trombose.

In de in dit proefschrift beschreven studies hebben we ons gericht op de betekenis van verlaagde vrij proteïne S spiegels. Het blijkt dat alleen sterk verlaagde vrij proteïne S spiegels (beneden het 5^{e} percentiel) een verhoogd risico op veneuze trombose geven. Deze klinisch belangrijke lage grens voor vrij proteïne S lijkt zowel voor erfelijk bepaalde proteïne S deficiënties als voor verworven vormen van verlaagd vrij proteïne S te gelden. Vaak wordt de ondergrens van een normaalwaardengebied als afkapwaarde voor het vaststellen van een deficiëntie gebruikt. Bij toepassing van de normaalwaardenondergrens als afkapwaarde voor vrij proteïne S zal echter in veel gevallen onterecht een proteïne S deficiëntie worden vastgesteld. We hebben laten zien dat de hoogste vrij proteïne S waarde die gevonden is in een groep met verhoogd tromboserisico en een genetisch bevestigde proteïne S deficiëntie, een betere afkapwaarde is. Door gebruik te maken van deze lagere afkapwaarde wordt de kans op het juist diagnosticeren van een klinisch relevante proteïne S deficiëntie sterk vergroot. Toch laten de vele studies, waarin gekeken is naar het tromboserisico bij patiënten met licht verlaagde vrij proteïne S spiegels, zeer verschillende resultaten zien. Recente studies hebben aangetoond dat de functie van proteïne S lijkt samen te hangen met die van TFPI. Verlaagde proteïne S waarden lijken daarbij gepaard te gaan met verlaagde TFPI spiegels. Zeer opvallend echter, vonden we juist verhoogde TFPI waarden in een subgroep met licht

verlaagde vrij proteïne S spiegels en een proteïne S Heerlen mutatie; een mutatie die geassocieerd is met een niet of slechts nauwelijks verhoogd tromboserisico. Het is aannemelijk dat de gevonden hoge TFPI spiegels het tromboserisico kunnen verlagen. Daarom stellen we voor om bij het vinden van een licht verlaagd vrij proteïne S tevens de TFPI spiegel te meten en hopen daarmee het tromboserisico voor patiënten met een proteïne S deficiëntie beter te kunnen inschatten. Toekomstig onderzoek zal echter moeten aantonen of de aanvullende TFPI meting nuttig is.

Appendix I

Dankwoord

"Knowledge is in the end based on acknowledgement."

Ludwig Wittgenstein (1889 - 1951)

In deze context wil ik graag een aantal mensen in het bijzonder bedanken voor hun directe of indirecte bijdrage bij de totstandkoming van dit proefschrift.

Prof. dr. J. van der Meer (1950-2009†). Beste Jan, ik had jou, als eerste promotor, graag willen bedanken voor de kans om als analist bij de Stolling te kunnen promoveren. Een mogelijkheid die niet vanzelfsprekend is, maar gelukkig met jouw vertrouwen in gang is gezet. Om deze reden richt ik mijn woorden tot Pyteke en de kinderen. Bedankt!

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René

Appendix II

List of publications

2012

Mulder R, ten Kate MK, Kluin-Nelemans HC, Mulder AB. PROS1 Heerlen polymorphism is associated with increased free plasma tissue factor pathway inhibitor levels.
Thromb Haemost. 2012;107:594-6.

2011

Mulder R, Tichelaar YI, Sprenger HG, Mulder AB, Lijfering WM. Relationship between cytomegalovirus infection and procoagulant changes in human immunodeficiency virus-infected patients.
Clin Microbiol Infect. 2011;17:747-9.

Mulder R, Tichelaar VY, Lijfering WM, Kluin-Nelemans HC, Mulder AB, Meijer K. Decreased free protein S levels and venous thrombosis in the acute setting, a case-control study.
Thromb Res. 2011;128:501-2.

Mahmoodi BK, Mulder AB, Waanders F, Spronk HM, **Mulder R**, Slagman MC, Vogt L, Navis G, Ten Cate H, Kluin-Nelemans HC, Laverman GD. The impact of antiproteinuric therapy on the prothrombotic state in patients with overt proteinuria.
J Thromb Haemost. 2011;9:2416-23.

2010

Mulder R, van Schouwenburg IM, Mahmoodi BK, Veeger NJ, Mulder AB, Middeldorp S, Kluin-Nelemans HC, Lijfering WM. Associations between high factor VIII and low free protein S levels with traditional arterial thrombotic risk factors and their risk on arterial thrombosis: results from a retrospective family cohort study.
Thromb Res. 2010;126:e249-54.

Mulder R, Ten Kate MK, Kluin-Nelemans HC, Mulder AB. Low cut-off values increase diagnostic performance of protein S assays.
Thromb Haemost. 2010;104:618-25.

2009

Lijfering WM, **Mulder R**, ten Kate MK, Veeger NJ, Mulder AB, van der Meer J. Clinical relevance of decreased free protein S levels: results from a retrospective family cohort study involving 1143 relatives.
Blood. 2009;113:1225-30.

2008

Ten Kate MK, Platteel M, **Mulder R**, Terpstra P, Nicolaes GA, Reitsma PH, van der Steege G, van der Meer J. PROS1 analysis in 87 pedigrees with hereditary protein S deficiency demonstrates striking genotype-phenotype associations.
Hum Mutat. 2008;29:939-47.

2006

Ten Kate MK, **Mulder R**, Platteel M, Brouwer JL, van der Steege G, van der Meer J. Identification of a novel PROS1 c.1113T-->GG frameshift mutation in a family with mixed type I/type III protein S deficiency.
Haematologica. 2006;91:1151-2.